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(54) Title: RESISTANCE AGAINST NEMATODES AND/OR APHIDS

(57) Abstract

The invention relates to genes capable of conferring resistance against nematodes and/or aphids. Preferred nucleic acids of the invention are DNA sequences which are at least part of the DNA sequence provided on figures or homologous thereto. The invention further relates to vectors, cells and seeds comprising said nucleic acids, as well as genetically transformed plants which are resistant to nematodes and/or aphids. The invention also relates to oligonucleotides, primers, diagnostic kit and polypeptides.

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RESISTANCE AGAINST NEMATODES AND/OR APHIDS

FIELD OF THE INVENTION

The present invention relates to resistance genes, DNA constructs, micro-organisms, plant cells and plants comprising said resistance genes. Furthermore the invention relates to genetically transformed plants which are resistant against nematodes and/or aphids. In addition, the invention relates to probes, and primers for the identification of the resistance genes and diagnostic kits comprising said probes and/or primers. Finally, the invention relates to polypeptides encoded by said resistance genes and the use of said polypeptides.

BACKGROUND OF THE INVENTION

Plant pathogens are responsible for substantially losses of plants and plant products due to infection of the plant. Plant diseases, as a result of infection by plant pathogens or pests, cause damage to the plants and/or plant products, reduce production and yield, limit the kind of plants that can grow in certain geographic areas and as a result cause severe (financial) losses to the grower.

Plant parasitic nematodes occur worldwide and most of them live most of their life in the topsoil layer. Although losses caused by direct feeding of nematodes on plant roots is considered to be of minor importance, several species, among them the root-knot nematodes belonging to the *Meloidogyne* species, the cyst nematodes belonging to the *Heterodera* species and *Globodera* species and other nematodes such as the *Nacobbus* species, cause severe damage and economic crop losses. Root-knot nematodes also occur throughout the world but are found more frequently and in greater numbers in areas with warmer climates and in greenhouses. The most important *Meloidogyne* species are *M. incognita, M. arenaria, M. hapla* and *M. javanica*, of which *M. hapla* also occurs in more temperate climatic zones.

Different means for control of the plant pathogens exist, such as mechanical cultivation of the soil, chemical treatment with pesticides, including nematicides and insecticides, or crop rotation. However, for certain plant pathogens, especially nematodes, these means of control are insufficient to protect the plants from

infection and resulting diseases. The only effective means of control involves plant host resistance (Russell, 1978, Plant Breeding for pest and disease resistance, Butterworths edit., 485 pp). The development of cultivars resistant to common plant pathogens is one of the major goals of plant breeders today, in order to reduce or ultimately eliminate the extensive need for pesticides. The burden for the environment of the large amounts of pesticides injected into the soil or sprayed on crops, trees etc. worldwide each year becomes too severe. Moreover, governmental regulations in Western countries restrict the use or even forbid the use of certain pesticides. Therefore, the need for plants which are resistant to one or more of their pathogens, or which have a reduced susceptibility to their attackers becomes more and more pressing. The development of resistant plants is one of the important objectives of current plant breeding programs. Plant genotypes susceptible for particular pathogens are crossed with resistant plant genotypes in order to introduce the resistant phenotype into the breeding line.

Damage by root-knot nematodes results primarily from the invasion of the plant roots by larvae which in a compatible relationship with the plant develop into a reproducing female. After invasion the larvae cause root cells to develop into giant cells on which they feed. Upon infection galls or knots are formed on the roots and the plant roots become otherwise disturbed, thickened and stunted. The root system thus disfunctions in the uptake of water and nutritional elements which damages the plant growth and development. Frequently damage to infected plants is increased by parasitic fungi attacking the weakened root tissue. Infected plants show reduced growth and smaller pale coloured leaves, with dwarf poor quality fruits or even without fruits, and tend to wilt in warmer climates (Agrios, 1988 in: Plant Pathology, Academic Press, Inc.). The damage and/or yield reduction caused by root-knot nematodes is substantial on the total agricultural production worldwide. In individual stand yield losses can be as high as 25-50 %, or even a crop may be killed.

In greenhouses root-knot nematodes can be controlled with steam sterilization of the soil or soil fumigation with nematicides. Under field conditions control can be achieved by the use of nematicides. However, the use of such, in

some cases very persistent, chemicals is increasingly debated and in some countries the use of certain nematicides is even forbidden.

Breeding genetically resistant genotypes is the most reliable and effective way of controlling root-knot disease. For a number of crop species the availability of resistance within the related germplasm has been reported, e.g. potato, cotton, tobacco, wheat, soybean, tomato, eggplant, common bean and alfalfa. Resistance breeding is hampered by firstly the limited occurrence of (known) resistance genes in the available germplasm, secondly, in some plant species the existence of crossing barriers between the cultivated crop species and the resistance bearing related species, and thirdly, screening tests for resistance versus susceptibility to nematodes are laborious and often not reliable. Therefore, resistance breeding is very difficult or not to achieve, or if possible time consuming.

Successful introduction of resistance genes has been realized in tomato. The resistance gene *Mi* (*Meloidogyne incognita*) has been introduced into cultivated tomato, *Lycopersicon esculentum*, after crossing with the related wild species *L. peruvianum* (PI.128657), using embryo culture. The *Mi* gene confers resistance to various *Meloidogyne* spp. (Fassuliotis, 1991, in: Genetic Improvement of Tomato, Springer Verlag edit.). The *Mi* resistance gene is reported to be a monogenic dominant gene (Gilbert and McGuire, 1956, Proc. Am. Soc. Hortic. Sci. **68**, 437-442) and is located on tomato chromosome 6. It is also postulated that the introgressed region comprising the *Mi* locus is involved in conferring resistance to potato aphid (*Macrosiphum euphorbia*) (Kaloshian *et al*, 1995, Proc. Natl. Acad. Sci. USA, **92**, 622-625).

Plants have developed a complex defense mechanism against attack and infection by pathogens. To date, the exact mechanism of their defense system is not yet elucidated.

Nematode resistance in tomato is expressed after penetration. After the juvenile larva enters the root and establishes itself at a feeding site, a hypersensitive reaction (HR) adjacent to the head of the nematode is triggered that results in local death of the host cells. The nematode is also adversely affected by this HR and dies (Fassuliotis, 1991, in: Genetic Improvement of Tomato, Springer Verlag edit.).

Wether or not there exists a gene-for-gene relationship sensu Flor (1956, Adv.Gen. 8, 29-54) as is frequently the case in other plant-pathogen relationships where resistance is based on HR-incompatibility is unknown.

The isolation of plant genes without knowing their gene products is very laborious and difficult, because of the enormous genome sizes of plant species: e.g. tomato has a genome size of 1000 Mb (10^9 base pairs of nuclear DNA), maize has a genome size of 3000 Mb and wheat has even more than 16 x 10^9 base pairs. Searching for a specific gene among these billions of base pairs is only feasible when (i) there are enough molecular markers tightly linked to the gene of interest and (ii) there is good genetic material available (Tanksley *et al.*, 1995, Trends in Genetics, **11**, p. 63-68).

Although, the isolation of a few resistance genes has been reported, none of these resistance genes are able to confer the host plant resistant to nematodes or to aphids. Examples of such isolated resistance genes are: *RPS2* from Arabidopsis (resistance to *Pseudomonas syringae* expressing avrRpt2), *N* from tobacco (resistance to tobacco mosaic virus), *Cf-9* from tomato (resistance to the leaf fungal pathogen *Cladosporium fulvum* carrying *avr9*) and L^6 from flax (resistance to the corresponding leaf rust fungal race) (Dangl, 1995, Cell **80**, 363-366).

The present invention provides the first isolated nematode resistance gene, and furthermore, provides the first isolated aphid resistance gene. Moreover, the present invention relates to a dual function resistance gene conferring reduced susceptibility to nematodes as well as aphids, and preferably to *Meloidogyne incognita* and *Macrosiphum euphorbiae* respectively.

SUMMARY OF THE INVENTION

The present invention relates to a nucleic acid comprising the *Mi* resistance gene which when present and expressed in a plant is capable of conferring to said plant resistance against nematodes and/or aphids. Furthermore, the invention relates to the *Mi* resistance gene of which the DNA sequence is disclosed herein. The invention also relates to a gene product encoded by the *Mi* resistance gene. In addition, the present invention relates to DNA constructs, cosmids, vectors, bacterial

strains, yeast cells and plant cells comprising the *Mi* resistance gene. In another aspect, the present invention relates to a genetically transformed plant, which is resistant to a nematode, said nematode being capable of infecting the untransformed plant. Furthermore, the invention relates to resistance genes which are homologous to the *Mi* resistance gene, and which, when present in a plant, are able of conferring said plant resistance to infection by pathogens.

Moreover, the present invention relates to a nucleic acid comprising the *Meu-1* resistance gene which when present in a plant is capable of conferring to said plant reduced susceptibility to aphids. In particular the *Meu-1* resistance gene corresponds to the *Mi* resistance gene. Especially the Meu-1 resistance gene has the same nucleotide sequence as the Mi resistance gene. Thus, the present invention also relates to genetically transformed plants, which are reduced susceptible, and preferably resistant to aphids, in particular to potato aphids.

Finally, the invention relates to oligonucleotides corresponding to the sequence of the said resistance gene or part thereof, and detection kits comprising said oligonucleotides.

DESCRIPTION OF THE FIGURES

Figure 1 shows a physical map of YAC 1/1172, YAC 2/1256 and YAC 1/1084, with a size of 570, 500 and 470 kb respectively. The position of the *Sfi*I and *Bss*HII restriction sites and the size of the restriction fragments are indicated. The location of the various AFLP markers on the restriction fragments are indicated.

Figure 2 shows a schematic drawing of the binary cosmid vector pJJ04541 which is used to construct a cosmid library of YAC 1/546. Plasmid pRK290 (20 kb large) (Ditta *et al*, 1980, Proc. Natl. Acad. Sci. USA, **77**, 7347-7351) was used as starting vector."Tet" refers to the gene conferring resistance to tetracyclin. "LB" signifies T-DNA left border repeat sequence, and "RB" signifies the right border repeat. The cauliflower mosaic virus 35S promoter sequence is indicated by "p35S", and "ocs3'" indicates the octopine synthase 3' end. "NPT" indicates neomycin phosphotransferase, and "cos" refers to the bacteriophage lambda cos site enabling

in vitro packaging. "pDBS" indicates the polylinker of pBluescript (Stratagene, La Jolla, CA, USA).

Figure 3A shows a schematic representation of the detailed position of the AFLP markers on YAC 1/1172, YAC 2/1256 and YAC 1/1084. Positioning is based on the cosmid contig constructed for the various defined regions.

Figure 3B shows a schematic representation of the cosmid contig of the region comprising the *Mi* resistance gene. The cosmids Mi-32, Mi-30, Mi-11, Mi-18, Mi-01 and Mi-14 are represented by horizontal lines. The location of the AFLP markers PM14 and PM25 is indicated.

Figure 4 shows a physical fine map of the cosmids Mi-32, Mi-30, Mi-11, Mi-18, Mi-01 and Mi-14 for the restriction enzyme *PstI*. The size of the *PstI* fragments is indicated (in kb). The *Mi* phenotype, as identified in an *in vitro* disease assay, of the R_0 plants comprising the various cosmids is indicated in the right end part of the figure. The DNA segment of which the nucleotide sequence was determined is indicated by a double line with a bidirectional arrow.

Figure 5 shows the nucleotide sequence of a DNA segment of approximately 9.9 kb around the AFLP marker PM14, and a deduced amino acid sequence of the *Mi* resistance gene. The initiation codon (ATG position 3263-3265) is underlined and the termination codon (TAG position 7109-7111) is double underlined, showing an open reading frame (ORF1) encoding a polypeptide of 1257 amino acids (fig. 7A). The Mi resistance gene comprises two intron sequences (shown in italics): one intron of 1306 nucleotides from position 1936 to position 3241 and one intron of 75 nucleotides from position 3305 to position 3379.

A second initiation codon (ATG position 3491-3493) which is in frame with the first initiation codon, results into a second open reading frame (ORF2) encoding a truncated polypeptide of 1206 amino acids (figure 7B).

The position of the AFLP marker PM14 is from nucleotide position 6921 (5'-TGCAGGA-3') to nucleotide position 7034 (5'-AGATTA-3').

Figure 6 shows a physical map of cosmids Mi-11 and Mi-18 and the determined nucleotide sequence of cosmid Mi-11. The sequence is divided in four contigs: con25 (5618 bp), con10 (898 kb), con62 (2495 bp) and Mi (9870 bp).

The lower part of the figure depicts the presence ("+") or absence ("-") of several PCR fragments, corresponding to parts of the DNA segment of Figure 5, which are represented as horizontal lines of different lengths at the right hand side of the table, in the various genetic backgrounds (YAC clone 2/1256, *E. coli* containing cosmid Mi-11, *A. tumefaciens* containing cosmid Mi-11, *E. coli* containing cosmid Mi-18, *A. tumefaciens* containing cosmid Mi-18, resistant tomato line E22, susceptible tomato line 52201, R_0 plants transformed with cosmid Mi-11 and R_0 plants transformed with cosmid Mi-18).

Nucleotide sequence of cosmid Mi-11 and cosmid Mi-18. Analysis of different contigs.

Figure 7 A: shows the deduced amino acid sequence of the polypeptide encoded by ORF1.

B: shows the deduced amino acid sequence of the truncated polypeptide encoded by ORF2.

Figure 8 depicts a schematic representation of the structure of the Mi-resistance gene.

Figure 9 depicts a schematic representation of the Mi-resistance gene family.

DETAILED DESCRIPTION OF THE INVENTION

In the description and examples that follow, a number of terms are used herein. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

- nucleic acid: a double-stranded DNA molecule. The nucleic acid can be genomic DNA, cDNA, synthetic DNA or any other DNA;
- oligonucleotide: a short single-stranded DNA molecule;
- primers: in general, the term primer refers to a single-stranded DNA molecule which can prime the synthesis of DNA;
- nucleic acid hybridization: a method for detecting related DNA sequences by hybridization of single-stranded DNA on supports such as nylon membrane or nitrocellulose filter papers. Nucleic acid molecules that have complementary

base sequences will reform the double-stranded structure if mixed in solution under the proper conditions. The double-stranded structure will be formed between two complementary single-stranded nucleic acids even if one is immobilized on a support. In a Southern hybridization procedure, the latter situation occurs;

- hybridization probe: to detect a particular DNA sequence in the Southern hybridization procedure, a labelled DNA molecule or hybridization probe is reacted to the fractionated DNA bound to a support such as nylon membrane or nitrocellulose filter paper. The areas on the filter that carry DNA sequences complementary to the labelled DNA probe become labelled themselves as a consequence of the reannealing reaction. The areas of the filter that exhibit such labelling can then be detected according to the type of label used. The hybridization probe is generally produced by molecular cloning of a specific DNA sequence or by synthesizing a synthetic oligonucleotide;
- homologous sequence: a sequence which has at least 50 %, preferably 60 %, more preferably 70 %, most preferably 80 % or even 90 % sequence identity with the particular sequence, whereby the length of sequences to be compared for nucleic acids is generally at least 120 nucleotides, preferably 200 nucleotides and more preferably 300 nucleotides and the length of sequences to be compared for polypeptides is generally at least 40 amino acid residues, preferably 65 amino acid residues and more preferably 100 amino acid residues. Alternatively, a homologous sequence refers to a sequence which can hybridize under stringent conditions to a particular sequence, and/or a DNA sequence coding for a polypeptide which has substantially the same properties as the polypeptide encoded by the particular DNA sequence, and/or a DNA sequence coding for a polypeptide having the same amino acid sequence as the polypeptide encoded by the particular DNA sequence and/or an amino acid sequence in which some amino acid residues have been changed with respect to the amino acid sequence of the particular polypeptide without substantially affecting the major properties of said polypeptide;

- stringent conditions refer to hybridization conditions which allow a nucleic acid sequence to hybridize to a particular sequence. In general, high stringent conditions refer to the hybridization conditions which allow a nucleic acid sequence of at least 50 nucleotides and preferably about 200 or more nucleotides to hybridize to a particular sequence at about 65 °C in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength, and washing at 65 °C in a solution comprising about 0,1 M salt, or less, preferably 0,2 x SSC or any other solution having a comparable ionic strength. These conditions allow the detection of sequences having about 90 % or more sequence identity. In general, lower stringent conditions refer to the hybridization conditions which allow a nucleic acid sequence of at least 50 nucleotides and preferably about 200 or more nucleotides to hybridize to a particular sequence at about 45 °C in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength, and washing at room temperature in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength. These conditions allow the detection of sequences having up to 50 % sequence identity. The person skilled in the art will be able to modify these hybridization conditions in order to identify sequences varying in identity between 50 % and 90 %;
- promoter: a transcription regulation region upstream from the coding sequence containing the regulatory sequences required for the transcription of the adjacent coding sequence and includes the 5' non-translated region or so called leader sequence of mRNA;
- terminator: a region downstream of the coding sequence which directs the termination of the transcription, also called the 3' non-translated region, which includes the poly-adenylation signal;
- resistance gene: a nucleic acid comprising a coding sequence as depicted in Figure 5, or part thereof, or any corresponding or homologous sequence;
- nematode(s): *Meloidogyne* spp. such as *Meloidogyne incognita*, *M. arenaria* or *M. javanica*, or any other genotype which is not able to infect a host having

a resistance gene according to the invention, such as but not limited to other root-knot nematodes, such as *M. hapla*, cyst nematodes such as *Heterodera* spp. or *Globodera* spp., or other nematodes such as *Nacobbus* spp., insects, such as potato aphid or any other plant pathogen or pest;

- resistance gene product: a polypeptide having an amino acid sequence as depicted in Figure 5, or part thereof, or any homologous amino acid sequence;
- R_0 plant: primary regenerant from a transformation experiment, also denoted as transformed plant or transgenic plant;
- R_1 line: the progeny of a selfed R_0 plant.
- R_2 line: the progeny of a selfed R_1 plant.
- R_1BC line: the progeny of a backcross between a R_1 plant and a plant of the genotype which was originally used for the transformation experiment.

In the present invention we have been able to identify and isolate the *Meloidogyne incognita* (*Mi*) resistance gene. The gene was cloned from a tomato genotype which is resistant to *Meloidogyne incognita*. The isolated *Mi* resistance gene according to the invention can be transferred to a susceptible host plant using Agrobacterium mediated transformation or any other known transformation method, and is involved in conferring to the host plant resistance against plant pathogens, especially to nematodes. The host plant can be tomato or any other genotype that is infected by said plant pathogen.

The present invention provides also a nucleic acid sequence comprising the *Mi* resistance gene, which is depicted in Figure 5.

With the *Mi* resistance gene according to the invention, one has an effective means of control against plant pathogens and/or pests, since the gene can be used for transforming susceptible plant genotypes thereby producing genetically transformed plants having a reduced susceptibility or being preferably resistant to a plant pathogen or pest. In particular, a plant which is genetically transformed with the

Mi resistance gene according to the invention has a reduced susceptibility to root-knot nematodes.

In a preferred embodiment the *Mi* resistance gene comprises the coding sequence provided in Figure 5 or any corresponding or homologous sequence or cDNA sequence, preceded by a promoter region and followed by a terminator region. The promoter region should be functional in plant cells and, preferably, corresponds to the native promoter region of the *Mi* resistance gene. However, it should be recognized that any heterologous promoter region can be used in conjunction with the coding sequences, as long as it is functional in plant cells. Preferably, a constitutive promoter is used, such as the CaMV 35 S promoter or T-DNA promoters, all well known to those skilled in the art. Furthermore, a suitable terminator region should be functional in plant cells all well known to those skilled in the art.

In addition the invention relates to the *Mi* resistance gene product which is encoded by the *Mi* resistance gene according to the invention and which has a deduced amino acid sequence provided in Figure 5 and Figure 7A, or which is homologous to the deduced amino acid sequence or part thereof. Furthermore, the *Mi* resistance gene product or a truncated polypeptide as provided in figure 7B can be used for raising antibodies against it, which antibodies can be used for the detection of the presence of the *Mi* resistance gene product.

In another aspect of the invention, the *Mi* resistance gene can be used for the design of oligonucleotides which are complementary to one strand of the DNA sequence as described in Figure 5, or part thereof, which can be used as hybridization probes, being accordingly labelled to allow detection, for the screening of genomic DNA or cDNA libraries for homologous genes. Homologous sequences which can hybridize to the probe under stringent hybridization conditions, and which encode for a gene product that is involved in conferring reduced susceptibility or resistance to a plant against a plant pathogen which normally infects said plant, are comprised within the scope of the present invention.

In another aspect of the invention oligonucleotides are designed based on the *Mi* resistance gene sequence, such that they can be used as hybridization probes in

Southern analysis. These probes can be used as molecular markers to distinguish plant genotypes having the resistance gene and plant genotypes lacking the resistance gene. Such a probe can be used as an additional tool in selection. In a preferred embodiment of the invention, oligonucleotides are designed based on the *Mi* resistance gene sequence, such that they can be used as primers in an amplification reaction, such as polymerase chain reaction (PCR), whereby the formation of an amplification product indicates the presence of the *Mi* resistance gene in a certain plant genotype. In a particular embodiment of the invention said primers direct the amplification of polymorphic fragments, so called molecular markers, which are closely linked to the *Mi* resistance gene. In a preferred embodiment said primers are used in selective restriction fragment amplification to identify AFLP markers, which are closely linked to the *Mi* resistance gene. The invention also relates to diagnostic kits, comprising oligonucleotides according to the invention, for the detection of the presence or absence of the *Mi* resistance gene within a genotype under study. Such a diagnostic kit circumvents the use of a laborious disease assay to screen for genotypes having the resistance gene or not.

Furthermore the invention relates to DNA constructs comprising a DNA sequence corresponding to the coding sequence of the *Mi* resistance gene and regulatory sequences functional in plant cells, said DNA sequence can be genomic DNA, cDNA, synthetic DNA or DNA of any other origin. Said regulatory sequences are either homologous or heterologous to the coding sequences of the *Mi* resistance gene. Preferably, said DNA construct comprises a nucleic acid whose sequence is provided in Figure 5, or part thereof.

The invention relates also to DNA constructs comprising the regulatory sequences, and more preferably the promoter region of the *Mi* resistance gene in conjunction with a structural gene sequence heterologous to said regulatory sequences.

The invention relates also to a DNA vector comprising a DNA construct according to the invention. Suitable vectors can be cloning vectors, transformation vectors, expression vectors, etc...., which are well known to those skilled in the art.

Furthermore, cells harbouring a vector comprising a DNA sequence corresponding to the sequence as described in Figure 5 or part thereof, or homologous thereto, are within the scope of the invention. Moreover, cells carrying a DNA construct according to the invention, are within the scope of this invention.

In one preferred embodiment of the invention, a genetically transformed plant is obtained by introducing the *Mi* resistance gene within the genome of said plant, being susceptible to nematodes, using standard transformation techniques, wherein said genetically transformed plant is resistant to nematodes.

In another embodiment of the invention, the *Mi* resistance gene can be transferred, using generally known transformation techniques, to a heterologous systems, such as but not limited to melon, tobacco, *Arabidopsis thaliana*, potato, sugarbeet, rapeseed, cucumber, pepper, eggplant. A heterologous system refers to a plant species which is different from the plant species from which the resistance gene was isolated.

In yet another embodiment of the invention, the *Mi* resistance gene corresponds to the *Macrosiphum euphorbiae (Meu-1)* resistance gene, and is involved in conferring to plants, transformed with the gene according to the invention, resistance to insects and in particular to aphids.

The DNA sequence comprising the *Mi* resistance gene as provided in the present invention has numerous applications of which some are described herein but which are not limiting the scope of the invention.

The present invention will be further described in detail in view of the isolation of the *Mi* resistance gene present in tomato lines which are resistant to root-knot nematodes. For the isolation of the *Mi* resistance gene we have used a map-based cloning (positional cloning) strategy, comprising the following steps:

(1) identification of molecular markers linked to the *Mi* resistance gene,

(2) construction of a high molecular weight genomic YAC library,

(3) physical mapping of the molecular markers on the YAC clones and YAC contig building,

(4) construction of a cosmid library of the YAC clones harbouring the linked molecular markers,

(5) physical fine mapping and cosmid contig building,

(6) genetic characterization of tomato mutants susceptible to root-knot nematodes,

(7) transformation of susceptible plants with the cosmids forming the contig,

(8) complementation analysis.

For the identification of molecular markers, we have used the selective restriction fragment amplification technology, hereinafter also denoted as AFLP™ technology, which randomly amplifies a subset of DNA fragments out of a complex mixture of many DNA fragments and said amplified fragments generate fingerprints that can be analyzed. In general, total DNA of different genotypes of the same plant species are subjected to the AFLP technology and the different AFLP fingerprints obtained from the different genotypes are compared. Fragments that are present in one genotype and absent in another genotype are polymorphic fragments and are denoted as AFLP markers. The selectivity in AFLP reactions is obtained by using randomly chosen selective nucleotides at the 3' end of the PCR primers immediately adjacent to the nucleotides of the restriction enzyme site. In an AFLP screening the DNA to be studied is subjected to different primer combinations. The total amount of different primers that can be used is determined by the number of selective nucleotides that are added to the 3' end (4 primers with 1 selective nucleotides, 16 primers with 2 selective nucleotides, 64 primers with 3 selective nucleotides). If two different restriction enzymes are used than there are twice the amount of primers. Those primers can be used in different combination. If all possible combinations are used in an AFLP screening, than all the fragments present should have been amplified with one of the primer combinations (Zabeau and Vos, EP 0534858).

For the identification of AFLP markers linked to the *Mi* resistance gene different tomato lines were subjected to an AFLP screening. In a first step, two sets of nearly isogenic lines for nematode resistance versus susceptibility were analyzed by AFLP fingerprinting using the following primers:

PstI-primers: 5'-GACTGCGTACATGCAGNN-3'

MseI-primers: 5'-GATGAGTCCTGAGTAANNN-3'

The N's indicate the variable selective nucleotides. In the AFLP screening all 16 primers possible for the *PstI*-primer and all 64 primers possible for the *MseI*-primer were used on the two sets of nearly isogenic lines, giving a total of 16 x 64 = 1024 tested primer combinations. Upon analysis of all the AFLP fingerprints a total of 30 candidate AFLP markers linked to the *Mi* resistance gene were identified. These candidate markers were subsequently tested on a panel of nematode resistant and nematode susceptible tomato lines for confirmation and distance of linkage to the *Mi* locus. The *Mi* resistance gene was introgressed in the cultivated tomato in 1944 from *Lycopersicon peruvianum*. Modern nematode resistant tomato lines have been subjected to numerous cycles of crossing expected to result in a small introgressed region from *Lycopersicon peruvianum* with the *Mi* resistance gene. Testing of the candidate AFLP markers on these modern tomato genotypes is expected to be a good test for assessing close linkage to the *Mi* locus. A panel of 7 resistant and 11 susceptible tomato genotypes was tested with the candidate AFLP markers. A total of 20 AFLP markers appeared to be present in all resistant lines and absent in all susceptible lines and are referred to as *Mi* linked AFLP markers.

Next, four of the AFLP markers were screened on a high molecular weight genomic library. The cloning of very large segments of DNA as large artificial chromosomes in yeast has become an essential step in isolating genes via positional cloning. The cloning capacity of the YAC vector allows the isolation of DNA fragments up to one million base pairs in length. The tomato line *Lycopersicon esculentum* E22, homozygous for the *Mi* locus, was used as source DNA to construct a YAC library. We obtained a YAC library containing 3840 clones with an average insert size of 520 Kb, representing approximately 2.2 genome equivalents of the tomato genome. Three positive YAC clones were obtained after the AFLP screening with the *Mi* linked AFLP markers: 1/1084, 1/1172 and 2/1256. Subsequently, the presence of all *Mi* linked AFLP markers was determined in the 3

YAC clones. All markers appeared present in one or more of the 3 YAC clones, which allowed a first positioning of the various *Mi* linked AFLP markers. The AFLP data indicated that the 3 YAC clones constituted an overlapping contig of approximately 1.4 Mb (see Figure 1).

To determine the physical size of the *Mi* locus comprising the *Mi* linked AFLP markers and comprised in YAC clones 1/1084, 1/1172 and/or 2/1256 a long-range restriction map of the YAC contig was constructed. This defined a DNA segment comprising the *Mi* locus of about 700 kb on which all the *Mi* linked AFLP markers were located (see Figure 1).

A size of 700 kb is still too large for direct localization of the *Mi* resistance gene. Such large inserts cannot be transformed into plant cells directly. Therefore, a cosmid library was constructed of the yeast strain containing YAC 1/1172 and a cosmid library was constructed of the yeast strain containing YAC 2/1256 using cosmid vectors which are suitable for *Agrobacterium* mediated transformation. The size of this binary cosmid vector amounts 29 kb and is shown schematically in Figure 2. The cloning capacity of this binary cosmid vector, using phage lambda packaging extract is within the range of 9 to 24 kb. Two banks of approximately 250,000 cosmid clones each were obtained from size fractionated yeast DNA. The cosmid banks were screened by colony hybridization using as probes labelled restriction fragments of the YACs. Positive cosmids clones were identified and in addition, the cosmids were grouped into seven defined regions covering the *Mi* region.

In the following step the set of cosmids of the seven defined regions were fingerprinted using restriction fragment amplification to determine their relative order. A cosmid contig covering a DNA segment of approximately 700 kb could be constructed. Subsequently, the presence of the *Mi* linked AFLP markers in this cosmid contig was determined. A physical map of the DNA segment comprising the *Mi* resistance gene with the positions of the various *Mi* linked AFLP markers was obtained (see Figure 3).

A total of 96 overlapping cosmids together constituted the DNA segment comprising the *Mi* resistance gene. Complementation analysis to identify the *Mi* resistance gene with such a large set of cosmids is a very laborious task. Therefore,

the position of the *Mi* resistance gene on the cosmid contig was determined using mutant tomato lines. These mutant lines are members from a family originating from a common ancestor and contained a wild-type (nematode resistant) *Mi* genotype but a mutant nematode susceptible phenotype. Upon analysis with the set of *Mi* linked AFLP markers on a large number of these mutant lines three *Mi* linked AFLP markers appeared to be absent in most mutants. These AFLP markers, therefore showed a good correlation between the AFLP *Mi* genotype and the *Mi* phenotype, in contrast to all other 17 AFLP markers. Two of these AFLP markers, PM14 and PM25 were adjacent, and the region around these markers was assumed to be the most likely position for the *Mi* resistance gene. A set of 6 overlapping cosmids defining a DNA segment of approximately 50 kb around AFLP markers PM14 and PM25 was selected for complementation analysis (see Figure 4).

The final step in the identification of the *Mi* resistance gene via positional cloning is the complementation of the corresponding susceptible phenotype. The 6 cosmids from the candidate *Mi* region were introduced in Agrobacterium tumefaciens through conjugative transfer in a tri-parental mating. The presence of the cosmid in the *A. tumefaciens* strains was determined comparing various restriction enzyme patterns as well as DNA fingerprints from the *A. tumefaciens* strains with the *E.coli* strain containing the cosmid. Only those *A. tumefaciens* cultures harbouring a cosmid with the same DNA pattern as the corresponding *E. coli* culture were used to transform a susceptible tomato line. A susceptible tomato line was transformed with cosmids Mi-32, Mi-30, Mi-11, Mi-18, Mi-01 and Mi-14 using standard transformation methods.

Roots of *in vitro* grown transformed R_0 plants were tested for disease symptoms in order to identify cosmids with the resistance gene. Root explants were transferred onto solidified medium in petri dishes and inoculated with ten galls from an axenic nematode culture of the root-knot nematode *Meloidogyne incognita*. Disease symptoms are scored six weeks after inoculation. A transgenic plant is considered resistant when no galls or one gall are visible on its root culture. A transgenic plant is considered susceptible when at least two galls have been induced on its root culture. The observations of the *in vitro* disease assay revealed

that 2 cosmids were able to complement the susceptible phenotype. The presence of the AFLP marker PM14 in the resistant R_0 plants indicated that the genomic insert present in cosmids Mi-11 and Mi-18 is also present in the R_0 plants and is involved in conferring the R_0 plants resistant to *Meloidogyne incognita*.

The primary regenerants (R_0 plants) of the transformation experiments were grown in the greenhouse for seed set to obtain R_1 lines which were tested for disease symptoms. The disease assay is performed on seedlings. Therefor, seeds are sown or small rooted plantlets are transferred into soil infected with *Meloidogyne incognita* and disease symptoms are scored 4 to 8 weeks after inoculation. Plants are considered to be resistant when three or less galls are visible on the roots. Plants are considered to be susceptible when more than three galls are formed on the roots. The observations of the *in vivo* disease assay revealed that the resistant R_0 plants are corresponding to cosmid Mi-11 transformants.

In order to confirm the stable integration of the *Mi* resistance gene into the genome of the transgenic R_0 plants, resistant plants of the R_1 lines were selfed and grown in the greenhouse for seed set to obtain R_2 lines. Seedlings of the R_2 lines were subjected to an *in vivo* nematode disease assay. The results obtained indicated the stable inheritance of the *Mi* resistance gene.

Finally, the inserts in cosmids Mi-11 and Mi-18 were further characterized. Sequencing analysis revealed a large open reading frame (ORF2) of 3621 nucleotides. The DNA sequence is listed in Figure 5.

The DNA sequence comprising the *Mi* resistance gene was further subjected to transcript mapping studies in order to determine the existence of intron sequences. These transcripts mapping studies were performed according to generally known methods whereby genomic DNA sequences are compared with cDNA sequences. The comparison of cDNA sequences and genomic sequences revealed the existence of two intron sequences in the *Mi* resistance gene. One intron of 1306 nucleotides is located from nucleotide position 1936 to 3241 and a second intron of 75 nucleotides is located from nucleotide position 3305 to 3379, as is depicted in Figure 5. The position of the transcription initiation site is postulated at or upstream of nucleotide 1880. The first ATG initiation codon is located at nucleotide

position 3263 which is 52 nucleotides upstream of the second intron, giving a large open reading frame (ORF1) encoding a polypeptide of 1257 amino acids (figure 7A).

Homology searches have shown that the polypeptides according to the invention belong to the LRR class of plant resistance proteins (Staskawicz et al, 1995, Science, 268, 661-667). In addition the protein can be divided into four regions designated A to D: region A comprises a high amount of leucine residues, region B comprises a nucleotide binding site motif, region C is the LRR region comprising 13 repeats with the following consensus sequence a–a-NL–L-a——a–a/S— (Jones and Jones, 1997, Advances in Botanical Research, 24, 89-167) and region D reveals no homology to any known protein.

For the identification and isolation of homologous sequences falling within the scope of the present invention, genomic and cDNA libraries were screened with the coding sequence of the *Mi* resistance gene as a probe under stringent hybridization conditions. Positive clones were isolated and used for complementation analysis.

Southern blot hybridizations on the YAC contig have been performed with an internal *PstI* fragment of the coding sequence of the *Mi* resistance gene. Three additional homologous regions could be identified: two in YAC 1/1172 and one in YAC 1/1084. Each region comprises 2 to 3 *Mi* homologues indicative of the fact that the *Mi* gene family is composed of about 10 to 12 members.

Surprisingly, aphid disease assays revealed that the R_0 plants, transformed with cosmid Mi-11, are resistant to *Meloidogyne incognita* as well as resistant to *Macrosiphum euphorbiae*, indicating that the genome insert present in cosmid Mi-11 is involved in conferring the R_0 plants resistant to nematodes as well as involved in conferring the R_0 plants resistant to aphids. In particular, a plant which is transformed with the resistance gene according to the invention has at least a reduced susceptibility to one or more pathogens, especially to root-knot nematodes and/or aphids.

In order to confirm the inheritance of the aphid resistance, (i) the previously obtained R_1 tomato lines which were derived from nematode resistant cosmid Mi-11 transformants, (ii) the R_2 lines derived from selfed nematode resistant R_1 plants and (iii) R_1BC lines obtained from nematode resistant R_1

plants backcrossed with susceptible tomato line 52201, were also tested for resistance against *M. euphorbiae*. The results obtained indicated the inheritance of the aphid resistance.

Cosmid Mi-11 was used for the transformation of nematode susceptible genotypes of tobacco and potato, according to general known transformation methods. Roots of *in vitro* grown transformed R_0 plants of tobacco and potato were tested for disease symptoms as previously described herein. The observations of the disease assay on the root cultures of the transformed plants indicated that the cosmid is involved in conferring to the transformed plants a reduced susceptibility to nematodes. The resistance gene according to the invention has an effect in reducing the susceptibility of a heterologous plant species to nematodes, preferably to *Meloidogyne spp.*, especially *Meloidogyne incognita*.

Furthermore tobacco transformants were also tested for aphid resistance, and resistant R_0 plants could be identified.

The resistance gene according to the invention has a dual function and has an effect in heterologous systems.

Cosmid Mi-11 has been deposited on August 5, 1996 as plasmid pKGMi-11 at Centraalbureau voor Schimmelcultures at Baarn, The Netherlands, under deposit number CBS 822.96.

Cosmid Mi-18 has been deposited on August 5, 1996 as plasmid pKGMi-18 at Centraalbureau voor Schimmelcultures at Baarn, The Netherlands, under deposit number CBS 821.96.

The following examples will provide a further illustration of the present invention which is nevertheless not limited to these examples.

EXAMPLES

EXAMPLE 1: DISEASE ASSAY

An axenic culture of the root-knot nematode *Meloidogyne incognita* is maintained on sterile roots of the tomato cultivar Moneymaker. The root cultures are grown on solidified B5 medium (Gamborg *et al* 1968, Experimental Cell Research 50: 151-158) with 2% saccharose and without hormones.

Root explants (1-5 cm), derived from *in vitro* grown transgenic tomato plants are transferred onto the solidified B5 medium mentioned above to start root cultures. At the same time each root explant is inoculated with ten galls from the axenic nematode culture. The galls are placed a few centimetres from the root explant. The Petri dishes with the roots and galls are incubated in the dark at 25°C. After four to six weeks the level of infection is determined by counting the number of galls formed on the root cultures.

The evaluation for resistance/susceptibility to *M. incognita* is as follows:

A transgenic plant is considered resistant when no or less than two galls are visible on its root culture. A transgenic plant is considered susceptible when at least two galls have been induced on its root culture.

EXAMPLE 2: IDENTIFICATION OF AFLP MARKERS LINKED TO A DNA SEGMENT COMPRISING THE *Mi* RESISTANCE GENE

Tomato lines (*Lycopersicon esculentum*)

A total of 9 tomato lines resistant to *Meloidogyne incognita* and 13 tomato lines susceptible to *M. incognita* were used to identify AFLP markers. Initially the AFLP screening was performed on two sets of nearly isogenic lines 83M-R (resistant) and 83M-S (susceptible), and Motelle (resistant) and Mobox (susceptible). The candidate markers resulting from this first screening were confirmed by a second screening on 7 *M. incognita* resistant and 11 *M. incognita* susceptible lines.

Two sets of nearly isogenic lines :

1.	83M-R	resistant	De Ruiter Zonen C.V., Bergschenhoek, The Netherlands (hereinafter "De Ruiter")
2.	83M-S	susceptible	De Ruiter
3.	Motelle	resistant	INRA, Montfavet, France
4.	Mobox	susceptible	INRA, Montfavet, France

The 7 *M. incognita* resistant lines and 11 *M. incognita* susceptible lines for confirmation :

5.	DR30	resistant	De Ruiter
6.	DR17	resistant	De Ruiter
7.	E22	resistant	Enza Zaden, de Enkhuizer Zaadhandel B.V., Enkhuizen, The Netherlands (hereinafter "Enza Zaden")
8.	E1	resistant	Enza Zaden
9.	DR6	resistant	De Ruiter
10.	DR10	resistant	De Ruiter
11.	1872	resistant	Royal Sluis B.V., Enkhuizen, The Netherlands (hereinafter "Royal Sluis")
12.	Moneymaker	susceptible	Agricultural University Wageningen
13.	DR12	susceptible	De Ruiter
14.	DR23	susceptible	De Ruiter
15.	GT	susceptible	De Ruiter
16.	RZ3	susceptible	Rijk Zwaan Zaadteelt en Zaadhandel B.V., De Lier, The Netherlands (hereinafter "Rijk Zwaan")
17.	RZ5	susceptible	Rijk Zwaan
18.	E3	susceptible	Enza Zaden
19.	E7	susceptible	Enza Zaden

20.	E16	susceptible	Enza Zaden
21.	RS1	susceptible	Royal Sluis
22.	RS2	susceptible	Royal Sluis

Isolation and modification of the DNA

Total tomato DNA from the 22 lines described above was isolated from young leaves as described by Bernatzki and Tanksley (Theor. Appl. Genet. 72, 314-321). The typical yield was 50 - 100 µg DNA per gram of fresh leaf material. Template DNA for AFLP analysis with the enzyme combination *Pst*I-*Mse*I was prepared as described by Zabeau and Vos (European Patent Application, EP 0534858), and is described briefly below:

0.5 µg of tomato DNA was incubated for 1 hour at 37°C with 5 units *Pst*I and 5 units *Mse*I in 40µl 10 mM Tris.HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA. Next 10 µl of a solution containing 5 pMol *Pst*I-adapters, 50 pMol *Mse*I-adapters, 1 unit T4 DNA-ligase, 1 mM ATP in 10 mM Tris.HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA was added, and the incubation was continued for 3 hours at 37°C. The adapters are depicted below:

The structure of the *Pst*I-adapter was:

5'-CTCGTAGACTGCGTACATGCA-3'
3'-CATCTGACGCATGT-5'

The structure of the *Mse*I-adapter was:

5'-GACGATGAGTCCTGAG-3'
3'-TACTCAGGACTCAT-5'

Adapters were prepared by adding equimolar amounts of both strands; adapters were not phosphorylated. After ligation, the reaction mixture was diluted to 500 µl with 10 mM Tris.HCl, 0.1 mM EDTA pH 8.0, and stored at -20°C. The diluted reaction mixture is further referred to as template DNA.

AFLP reactions

The primers used for the AFLP screening are depicted below:

*Pst*I-primers:	5'-GACTGCGTACATGCAGNN-3'
*Mse*I-primers:	5'-GATGAGTCCTGAGTAANNN-3'

The N's in the primers indicate that this part of the primers was variable. In the AFLP screening all 16 possible primers were used for the *Pst*I-primer and all 64 possible primers were used for the *Mse*I-primer. This gave a total of 16 x 64 combinations of *Pst*I- and *Mse*I-primers, is 1024 primer combinations. All 1024 primer combinations were used in the AFLP screening for *Mi* linked AFLP markers. The AFLP reactions were performed in the following way:

AFLP reactions employed a radio-actively labelled *Pst*I-primer and a non-labelled *Mse*I-primer. The *Pst*I-primers were end-labelled using (γ-^{33}P)ATP and T4 polynucleotide kinase. The labelling reactions were performed in 50 µl 25 mM Tris.HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM spermidine.3HCl using 500 ng oligonucleotide primer, 100 µCi (γ-^{33}P)ATP and 10 units T4 polynucleotide kinase. For AFLP analysis 20 µl reaction mixture were prepared containing 5 ng labelled *Pst*I-primer (0.5 µl from the labelling reaction mixture), 30 ng *Mse*I-primer, 5 µl template-DNA, 0.4 units Taq-polymerase, 10 mM Tris.HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of all 4 dNTPs. AFLP reactions were performed using the following cycle profile: a 30 seconds DNA denaturation step at 94°C, a 30 seconds annealing step (see below), and a 1 minute extension step at 72°C. The annealing temperature in the first cycle was 65°C, was subsequently reduced each cycle by 0.7°C for the next 12 cycles, and was continued at 56°C for the remaining 23 cycles. All

amplification reactions were performed in a PE-9600 thermocycler (Perkin Elmer Corp., Norwalk, CT, USA).

Gel analysis of AFLP reaction products

After amplification, reaction products were mixed with an equal volume (20 µl) of formamide dye (98% formamide, 10 mM EDTA pH 8.0, and bromo phenol blue and xylene cyanol as tracking dyes). The resulting mixtures were heated for 3 minutes at 90°C, and then quickly cooled on ice. 2 µl of each sample was loaded on a 5% denaturing (sequencing) polyacrylamide gel (Maxam and Gilbert, Methods in Enzymology 65, 499-560). The gel matrix was prepared using 5% acrylamide, 0.25% methylene bisacryl, 7.5 M urea in 50 mM Tris/50 mM Boric acid/1 mM EDTA. To 100 ml of gel solution 500 µl of 10% APS and 100 µl TEMED was added and gels were cast using a SequiGen 38 x 50 cm gel apparatus (Biorad Laboratories Inc., Hercules, CA, USA). Sharktooth combs were used to give 97 lanes on the SequiGen gel units. 100 mM Tris/100 mM Boric acid/2 mM EDTA was used as running buffer. Electrophoresis was performed at constant power, 110 Watts, for approximately 2 hours. After electrophoresis, gels were fixed for 30 minutes in 10% acetic acid dried on the glass plates and exposed to Fuji phospho image screens for 16 hours. Fingerprint patterns were visualized using a Fuji BAS-2000 phospho image analysis system (Fuji Photo Film Company Ltd, Japan).

AFLP screening for linked markers

An AFLP screening was performed using all possible 1024 *PstI-MseI* primer combinations on the two sets of nearly isogenic lines. The aim was to identify AFLP markers present in both resistant lines and absent in both susceptible lines. AFLP gels contained the AFLP fingerprints of 24 primer combinations of the 4 isogenic lines, giving a total of 43 gels. A total of 30 AFLP markers were identified present in both resistant lines and absent in both susceptible lines. These markers are referred to as candidate *Mi* linked AFLP markers.

Next, AFLP reactions were performed to determine the presence of the 30 candidate markers on the 7 resistant and 11 susceptible tomato lines. Of the 30 candidate

markers 20 markers appeared to be present in the 7 resistant lines and absent in the 11 susceptible lines. These 20 markers were used in further studies to map the *Mi* resistance gene. The primer combinations required to identify the 20 *Pst*I-*Mse*I markers are depicted in Table 1. In the column with the primer combinations, "*Pst*I-" refers to the sequence 5'-GACTGCGTACATGCAG-3' and "*Mse*I-" refers to the sequence 5'-GATGAGTCCTGAGTAA-3'. For example, marker PM14 can be identified using the *Pst*I-primer having the following sequence: 5'-GACTGCGTACATGCAGGA-3', and the *Mse*I-primer having the following sequence: 5'-GATGAGTCCTGAGTAATCT-3.'

TABLE 1

marker	primer combination with selective extensions (NN/NNN)
PM02	*Pst*I-AT /*Mse*I-AAA
PM07	*Pst*I-AA /*Mse*I-TAC
PM08	*Pst*I-CT /*Mse*I-ACT
PM10	*Pst*I-CA /*Mse*I-TCT
PM11	*Pst*I-TA /*Mse*I-TGA
PM13	*Pst*I-GA /*Mse*I-ATC
PM14	*Pst*I-GA /*Mse*I-TCT
PM15	*Pst*I-GT /*Mse*I-GAC
PM16	*Pst*I-GT /*Mse*I-TCT
PM17	*Pst*I-AT /*Mse*I-AAG
PM18	*Pst*I-AT /*Mse*I-TAG
PM19	*Pst*I-GG /*Mse*I-ATT
PM20	*Pst*I-TG /*Mse*I-AAT
PM21	*Pst*I-TG /*Mse*I-TTT
PM22	*Pst*I-TG /*Mse*I-GCT
PM23	*Pst*I-GT /*Mse*I-GAA
PM24	*Pst*I-AA /*Mse*I-CTG

PM25	*Pst*I-AC /*Mse*I-GTG
PM27	*Pst*I-AA /*Mse*I-CTA
PM29	*Pst*I-TA /*Mse*I-GGA

EXAMPLE 3: CONSTRUCTION AND SCREENING OF A TOMATO YAC LIBRARY

Material

The tomato line *Lycopersicon esculentum* E22 (Enza Zaden) homozygous for the *Mi* locus, was used as source DNA to construct a YAC library. Protoplasts were isolated from the leaves of *in vitro* shoots which were two to three weeks old as described by Van Daelen *et al* (Plant Mol. Biol. **12**, 341-352).

Viable protoplasts (concentration of 50 million protoplasts per ml) were collected and mixed with an equal volume of agarose (1%, Seaplaque, FMC Bioproducts, Rockland, Maine, USA) to form a plug. The protoplasts embedded into the plugs were lysed with lysis mix (0.5 M EDTA, 1% N-Laurylsarcosinate and 1 mg/ml proteinase K, pH= 8.0). After lysis, the plugs were stored at 4°C in storage buffer (fresh lysis mix) until used. Approximately 3 million protoplasts per plug, to obtain about 4.5 µg of chromosomal DNA were used for further studies. Plasmid pYAC4 containing an unique *Eco*RI cloning site was used as cloning vector and the yeast strain AB1380 was used as a host (Burke *et al*, Science **236**, 806-812).

YAC library construction

High molecular weight DNA isolation, partial digestion with *Eco*RI in the presence of *Eco*RI methylase, ligation of vector arms to genomic DNA, size selection by pulsed field gel electrophoresis and transformation of the yeast host was performed as described by Burke *et al*, (Science **236**, 806-812) and Larin *et al*, (Proc Natl Acad Sci USA **88**, 4123-4127).

All standard manipulations were carried out as described in Molecular cloning: a laboratory manual by Sambrook *et al*, (Cold Spring Harbor Laboratory Press).

3840 clones with a average insert size of 520 kb, which corresponds to 2.2 genome equivalents were finally obtained and the individual clones were stored in 40 96-wells microtiter plates containing 75 µl YPD solution (1% yeast extract, 2% peptone and 2% dextrose).

Screening YAC library

To reduce the number of samples handled, the cells of one 96-well microtiter plate were pooled (a platepool) and used for DNA isolation as described by Ross *et al* (Nucleic Acids Res., **19**, 6053). The 2.2 genome equivalent tomato YAC library consists of 40 96-wells microtiter wells and as a result DNA of the 40 platepools were screened with the AFLP markers PM10, PM13, PM21 and PM25 using the AFLP protocol as described in Example 2. PM10, PM13, PM21 and PM25 were selected to screen the YAC platepools because these markers do not interfere with the background bands of the yeast strain AB1380. Three positive platepools out of the 40 were identified with these four AFLP markers as shown in Table 2. Subsequently, a secondary screening with the four AFLP markers (PM10, PM13, PM21 and PM25) of the 96 individual YAC clones of each plate was employed to find the correct address of the YAC clones. Three individual YAC clones were identified, designated 1/1084, 1/1172 and 2/1256 (Table 2). Subsequently, the three individual YAC clones were analyzed with the remaining AFLP markers. All of the identified markers PM02 to PM29 were present on one or more these three YAC clones (Table 3). The size of the YAC clone was determined by Pulse-field gel electrophoretic (PFGE) analysis using contour-clamped homogeneous electric field (CHEF; Chu *et al* Science, **235**, 1582-1585) and appeared to be 470 kb (1/1084), 570 kb (1/1172), and 500 kb (2/1256) respectively.

TABLE 2

Platepool nr	PM10	PM13	PM21	PM25	YAC detected (size in kb)
2	-	-	+	-	YAC 1/1172 (570 kb)
16	+	+	-	+	YAC 2/1256 (500 kb)
4	-	+	-	-	YAC 1/1084 (470 kb)

TABLE 3

Marker	1/1172	2/1256	1/1084
PM02	-	-	+
PM07	-	+	-
PM08	-	+	+
PM10	-	+	-
PM11	-	+	-
PM13	-	+	+
PM14	+	+	-
PM15	-	+	-
PM16	+	-	-
PM17	-	+	-
PM18	-	+	+
PM19	-	+	-
PM20	-	+	-
PM21	+	-	-
PM22	-	+	+
PM23	-	+	-
PM24	-	+	-

30

PM25	-	+	-
PM27	-	+	-
PM29	-	+	-

EXAMPLE 4: CONSTRUCTION OF A LONG RANGE PHYSICAL MAP OF THE *Mi* YAC CONTIG AND LOCATION OF THE AFLP MARKERS

The 3 YAC clones 1/1172, 2/1256 and 1/1084 were subjected to partial digestion with increasing concentration of the restriction enzymes *Sfi*I and *Bss*HII. The samples were fractionated by PFGE, transferred to a Gene Screen Plus membrane (DuPont NEN, Boston, MA, USA) and assayed by hybridization using end-adjacent sequence probes according to the protocol for indirect end-label mapping as described by Burke *et al* (Science **236**, 806-812). A physical map of YAC 1/1172, 2/1256 and 1/1084 for the enzymes *Sfi*I and *Bss*HII could be constructed as shown in Figure 1. The overlap between the various YAC clones was determined by Southern blot analysis using the obtained restriction fragments as a probe on digest of the three YAC clones. A YAC contig with a size of 1.4 Mb could be constructed. In order to isolate the YAC fragments the digests were run on PFGE. Digestion of YAC 1/1172 with *Sfi*I resulted in two fragments (200 Kb and 370 Kb). Digestion of YAC 2/1256 with *Bss*HII resulted in four fragments (40 Kb, 90 Kb, 110 Kb and 260 Kb) whereas digestion of YAC 1/1084 with *Bss*HII gave two fragments with a size of 70 and 400 kb. As a result the 1.4 Mb YAC contig could be dissected into 8 regions corresponding to the 8 restriction fragments obtained from the three YAC clones, covering the complete *Mi* region and adjacent sequences.

To position the various AFLP markers within these 8 regions on the physical map, the AFLP markers were used as hybridization probes on the partial and complete *Sfi*I and *Bss*HII digests of YAC clones 1/1172, 2/1256 and 1/1084. Therefore, each AFLP marker fragment was excised from the dried gel and eluted by means of diffusion in a buffer containing 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA (pH=8.0), 0.1% SDS, re-amplified with the corresponding

unlabelled AFLP primers and, subsequently labelled with ^{32}P according to the random primer method of Feinberg and Vogelstein (Anal. Biochem. **132**, 6-10). Each AFLP marker could be assigned to one or more of the eight regions as outlined in Table 4 and Figure 1.

TABLE 4

YAC fragment	*Mi* linked AFLP markers detected by hybridization
200 kb *Sfi*I-fragment 1/1172	-
370 kb *Sfi*I-fragment 1/1172	PM14, PM16, PM21
260 kb *Bss*HII-fragment 2/1256	PM10, PM11, PM17, PM19, PM23, PM24, PM29
90 kb *Bss*HII-fragment 2/1256	PM07, PM27
110 kb *Bss*HII-fragment 2/1256	PM08, PM13, PM14, PM15, PM20, PM22, PM25
40 kb *Bss*HII-fragment 2/1256	PM18
70 kb *Bss*HII-fragment 1/1084	PM08, PM13, PM22
400 kb *Bss*HII-fragment 1/1084	PM02, PM18

EXAMPLE 5: CONSTRUCTION OF A COSMID LIBRARY OF YAC CLONES 1/1172 AND 2/1256

Material

The binary cosmid vector pJJ04541 is a derivative of pJJ1881 (Jones *et al*, Transgenic Research **1**, 285-297) and is based on plasmid pRK290 containing the tetracyclin resistance gene for selection in *Escherichia coli* and *Agrobacterium tumefaciens*. Into the unique *Eco*RI site of pRK290, T-DNA carrying sequences (LB; left border repeat, RB signifies the right border repeat) that flank

- the cos site of bacteriophage lambda

- the neomycin phosphotransferase gene (Beck *et al*, Gene **19**, 327-336) whose expression is driven by the cauliflower mosaic virus 35S promoter sequence (Odell *et al*, Mol Gen Genet **223**, 369-378), and
- the pBluescript (Stratagene, La Jolla, California, USA) polylinker sequence.

The size of pJJ04541 amounts 29 kb and is shown schematically in Figure 2. The cloning capacity of this binary cosmid vector, using phage lambda packaging extracts is within the range of 9 to 24 kb.

Library construction

Total DNA of the *Saccharomyces cerevisae strain* AB1380 containing YAC 1/1172 and total DNA of the *Saccharomyces cerevisae strain* AB1380 containing YAC 2/1256 was isolated using zymolyase to make protoplasts according to Green and Olsen (Proc Natl Acad Sci USA **87**, 1213-1217).

An aliquot of both DNAs was analyzed on PFGE. Both DNA isolates appeared to have a size of ≥100 kb.

Approximately 15 µg of each DNA was partially digested with *Sau*3A generating molecules with an average size of 15-25 kb. Subsequently, the samples were centrifugated through a 10-35% sucrose gradient for 22 hours, 22.000 rpm at 20°C in a Beckman SW41 rotor. 0.5 ml fractions were collected using a needle pierced through the bottom of the centrifuge tube. An aliquot of these fractions was analyzed on a 0.7% agarose gel. The fractions containing DNA molecules with a size of ≈20 kb were pooled and concentrated by ethanol precipitation.

Subsequently, the cohesive ends were partially filled-in with dATP and dGTP using the strategy of partial filling of 5'-extensions of DNA produced by type II restriction endonuclease as described by Korch (Nucleic Acids Res. **15**, 3199-3220) and Loftus *et al* (Biotechniques **12**, 172-176).

The binary cosmid vector pJJ04541 was digested completely with *Xho*I and the linear fragment was partially filled-in with dTTP and dCTP as described by Korch (Nucleic Acids Res. **15**, 3199-3220).

The 20-kb fragments were ligated to the cosmid vector and transduced to *E. coli* strain XL1-Blue MR (Stratagene, La Jolla, California, USA) using phage lambda

Gigapack II XL packaging extracts (Stratagene, La Jolla, California, USA) as recommended by the manufacturers. Selection was performed on LB (1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl, pH 7.5) agar plates containing 10 mg/l of tetracyclin. Two banks of approximately 250.000 cosmid clones per bank were made from 2-3 µg of size fractionated yeast DNA of YAC clones 1/1172 and 2/1256 respectively.

Subsequently, these transformants were stored into the wells of microtiter plates (96-wells, 100 µl of LB medium containing 10 mg/l of tetracyclin). Replicas of the 96-well grid of cosmid clones in microtiter plates were stamped onto Gene Screen Plus membrane filters (NEN Dupont) and allowed to grow into colonies on media. Colony hybridization, as described by Sambrook *et al* (in: Molecular cloning: a laboratory manual, 1989, Cold Spring Harbor Laboratory Press), using ^{32}P-labelled YAC clones 1/1172 and 2/1256 revealed positive cosmids. Of about 10.000 colonies of YAC 1/1172 approximately 200 positive cosmid clones were identified. Of about 20.000 colonies of YAC 2/1256 300 positive cosmid clones were identified.

EXAMPLE 6: FINE MAPPING OF THE *Mi* RESISTANCE GENE SEGMENT AND POSITIONING OF THE AFLP MARKERS

Dividing the cosmids in defined regions

In order to divide the cosmids into seven defined regions, the 200 positive cosmid clones of YAC 1/1172 and the 300 positive cosmid clones of YAC 2/1256 were hybridized with 7 of the 8 restriction fragments (YAC fragments) as outlined in Example 4 (see Table 4 and Figure 1). Positive cosmids for each of the 7 YAC fragments were identified. In addition, cosmids could be identified which reacted positively with the overlapping restriction fragments of the two different YAC clones.

Construction of a cosmid contig of the *Mi* resistance gene segment

In order to construct a cosmid contig of all the positive identified cosmids in the various defined regions restriction fragment amplification was used. Approximately 500 ng of each cosmid was used for template preparation and the primers in the

amplification of restriction fragments were a *EcoR*I-primer 5'-GACTGCGTACCAATTC-3' having no selective nucleotides and a *Mse*I-primer 5'-GATGAGTCCTGAGTAA-3' having no selective nucleotides according to the method as described in Example 2. The *EcoR*I-primer was labelled at the 5' end and all the 500 cosmids were amplified using *EcoRI/Mse*I-primer set. The DNA fingerprints contained about 8 to 20 amplified fragments. Sets of cosmids containing amplified fragments of identical size were selected from each region and were rerun on polyacrylamide gels as described in Example 2 until a contiguous array of all the amplified fragments throughout the defined regions could be constructed. In addition, the cosmid contig of one region was aligned with the adjacent regions in order to construct a cosmid contig of the *Mi* locus. In this way a cosmid contig of 96 cosmids was constructed spanning the *Mi* locus of approximately 800 kb.

Detailed positioning of the *Mi* linked AFLP markers on the cosmid contig

In order to position the 20 *Mi* linked AFLP markers on the cosmid contig, the 96 cosmids were digested with *Pst*I followed by Southern blot analysis according to Southern, J. Mol. Biol. **98**, 503-515.

The AFLP markers were used as hybridization probes as described in Example 4 on the Southern blot of the 96 *Pst*I digests of the cosmids. The exact position of the *Mi* linked AFLP markers, except marker PM02, is outlined in Figure 3A.

EXAMPLE 7: GENETIC ANALYSIS OF *Mi* MUTANTS

A family of mutant tomato lines was made available through Enza Zaden. These lines were derived from a F_1 hybrid heterozygous for the *Mi* resistance gene and heterozygous for the *Aps-1* gene (encoding acid phosphatase-1), which is very closely linked to *Mi* (Stevens and Rick, 1986, in: The Tomato Crop, Atherton & Rudich edit., Chapman and Hall, p. 35-109). Different alleles of the *Aps-1* gene can be determined by isozyme analysis (Vallejos, 1983, in: Isozymes in plant genetics and breeding, Tanksley and Orton edit., part A, Elsevier, Amsterdam, 469-515) The *Aps-1*^{1} allele originates from *L. peruvianum* and has been introgressed into several

nematode resistant tomato genotypes by co-segregation with the *Mi* resistance gene. A scheme of these mutant lines is depicted below:

F_1 - hybrid (heterozygous *Aps-1*, *Mi* resistant phenotype)

↓ *selfed*

F_2 - lines (segregating *Aps-1* 1:2:1, segregating *Mi* resistance 3:1)

↓ *selfing of heterozygous (Aps-1) F_2 plants*

F_3 - lines (segregating *Aps-1* 1:2:1, segregating *Mi* resistance 3:1)

↓ *selfing of heterozygous (Aps-1) F_3 plants*

F_4 - lines (segregating *Aps-1* 1:2:1, segregating *Mi* resistance 3:1)

↓ *selfing of heterozygous (Aps-1) F_4 plants*

F_5 - lines (segregating *Aps-1* 1:2:1, *Mi* susceptible)

↓ *selfing of heterozygous (Aps-1) F_5 plants*

F_6 - lines (segregating *Aps-1* 1:2:1, *Mi* susceptible)

↓ *selfing of homozygous (Aps-1^1) F_6 plants*

F_7 - lines (all *Aps-1^1*, *Mi* susceptible)

↓ *selfing of homozygous (Aps-1^1) F_7 plants*

F_8 - lines (all *Aps-1^1*, *Mi* susceptible)

In the F_1, F_2, F_3 and F_4 lines of this family the presence of the *Aps-1^1* allele correlates with the *Mi* resistant phenotype, whereas absence of the *Aps-1^1* allele correlates with the *Mi* susceptible phenotype. In the F_5 and subsequent progenies this correlation is lost: all plants are susceptible to nematodes regardless of the *Aps-1* alleles.

Twenty individuals from each F_2, F_3, F_4, F_5, F_6, F_7 and F_8 generation were tested for nematode resistance, for presence of the *Aps-1* allele and presence of the *Mi* linked AFLP markers. Nematode testing of seedlings was performed in soil contaminated with root galls of *M. incognita*. The nematode resistance results were as indicated in the above scheme: 3:1 segregation in F_2, F_3 and F_4 plants and susceptibility in F_5 and progeny populations. Most of the *Mi* linked AFLP markers indicated an identical *Mi* genotype as the *Aps-1* isozyme marker. However, 3 of the AFLP markers PM14,

PM16 and PM25 appeared to segregate with the *Mi* phenotype: In most F_5, F_6, F_7 and F_8 plants the *Mi* susceptibility was indicated by the absence of these markers. The AFLP markers PM14, PM16 and PM25 showed a correlation between the AFLP *Mi* genotype and *Mi* phenotype in the mutants. Markers PM14 and PM25 are adjacent on the physical map as shown in Figure 3B, and therefore, it was postulated that the region surrounding these AFLP markers was a good candidate to comprise the *Mi* resistance gene.

EXAMPLE 8: PHYSICAL MAP OF THE OVERLAPPING COSMID CLONES COMPRISING THE *Mi* RESISTANCE GENE

The identification of cosmids hybridizing with the *Mi* linked AFLP markers PM14 and PM25 was performed in Example 6. PM14 identifies cosmids Mi-11, Mi-18 and Mi-01 whereas PM25 identifies cosmids Mi-18 and Mi-01.

Subsequently, a small cosmid array around cosmids Mi-11, Mi-18 and Mi-01 was selected from the cosmid contig described in Example 6. A contig of 6 cosmids comprising the 3 identified cosmids and the adjacent cosmids, was selected. These 6 cosmids are Mi-32, Mi-30, Mi-11, Mi-18, Mi-01 and Mi-14. In order to make a physical fine map of these 6 cosmids, the DNA samples of the cosmid contig were digested with *PstI* followed by electrophoresis on a 0.8%-agarose gel. The physical overlap between the various cosmids could be determined. Combining these data with the data obtained about the detailed positioning of the *Mi* linked AFLP markers on the cosmid contig (see Example 6) a physical fine map with the location of PM14 and PM25 could be constructed as shown in Figure 4. The cosmid contig around the AFLP markers PM14 and PM25 was calculated to be approximately 50 kb.

EXAMPLE 9: TRANSFORMATION

Transfer of cosmids to *Agrobacterium tumefaciens*

The cosmid clones Mi-32, Mi-30, Mi-11, Mi-18, Mi-01, Mi-14 and the control cosmid pJJ04541 were introduced in *Agrobacterium tumefaciens* through conjugative transfer in a tri-parental mating with helper strain HB101 (pRK2013) essentially

according to Deblaere *et al* (Methods in Enzymology **153**, 277-292). *E.coli* were grown in LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl, pH 7.5) supplemented with 5 mg/l tetracyclin at 37°C. The helper strain HB101 (pRK2013) was grown under identical conditions in LB medium supplemented with 100 mg/l kanamycin sulphate.

Agrobacterium tumefaciens strain AGL1 (Lazo *et al*, Bio/Technology, **9**, 963-971, 1991) was grown in LB medium supplemented with 100 mg/l carbenicillin at 28°C. Overnight cultures were diluted 1:100 in LB medium without any antibiotics and after 6 hours of growth, 0.1 ml each of the *Agrobacterium* culture, the helper strain culture and a cosmid strain culture were mixed and plated on LB agar plates without antibiotics. After overnight incubation at 28°C, the mixture was plated on LB medium agar plates containing 100 mg/l carbenicillin and 10 mg/l tetracyclin to screen for transconjugants. Plates were incubated for 3-4 days at 28°C. Two serial passages through selective agar plates were performed to select for single transconjugant *Agrobacterium* colonies.

Characterization of A. tumefaciens transconjugants

Small-scale cultures were grown from selected colonies and grown in LB medium containing 10 mg/l tetracyclin. Plasmid DNA was isolated by alkaline lysis using the method as described by Ish-Horowicz et al (Nucl. Acids Res. 9, 2989-2997, 1981), and digested with *Bgl*II using standard techniques. In addition, restriction fragment amplification on miniprep DNA of *A. tumefaciens* was performed using the enzyme combination *Eco*RI/*Mse*I and primers having no selective nucleotide as described in Example 6. Subsequently, the *Bgl*II restriction enzyme pattern as well as the DNA fingerprint of the *A. tumefaciens* transconjugant were compared with those of miniprep DNA of the *E. coli* strain containing the cosmid. Only those *A. tumefaciens* transconjugants harbouring a cosmid with the same DNA pattern as the corresponding *E. coli* culture were used to transform a susceptible tomato line.

Transformation of a susceptible tomato line

Seeds of the susceptible tomato line 52201 (Rijk Zwaan, De Lier, The Netherlands) were surface-sterilized in 2% sodium hypochlorite for 10 minutes, rinsed three times in sterile distilled water, and placed on germination medium (consisting of half-strength MS medium according to Murashige and Skoog, Physiol. Plant. **15,** 473-497, with 1% (w/v) sucrose and 0.8% agar) in glass jars or polypropylene culture vessels. They were left to germinate for 8 days. Culture conditions were 25°C, a photon flux density of 30 $\mu mol.m^{-2}.s^{-1}$ and a photoperiod of 16 /24 h.

Transformation of tomato was performed according to Koornneef *et al* **(1986),** In: Tomato Biotechnology, 169-178, Alan R. Liss, Inc., and is described briefly below. Eight day old cotyledon explants were precultured for 24 hours in Petri dishes containing a feeder layer of *Petunia hybrida* suspension cells plated on MS20 medium (culture medium according to Murashige and Skoog, **(1962)** Physiol. Plant. **15,** 473-497 with 2% (w/v) sucrose and 0.8% agar) supplemented with 10.7 μM α-naphthaleneacetic acid and 4.4 μM 6-benzylaminopurine. The explants were then infected with the diluted overnight culture of *Agrobacterium tumefaciens* containing the cosmid clones Mi-32, Mi-30, Mi-11, Mi-18, Mi-01 and Mi-14 or the cosmid vector pJJ04541 for 5-10 minutes, blotted dry on sterile filter paper and cocultured for 48 hours on the original feeder layer plates. Culture conditions were as described above. Overnight cultures of *Agrobacterium tumefaciens* were diluted in liquid MS20 medium (medium according to Murashige and Skoog (1962) with 2% (w/v/) sucrose, pH 5.7) to an $O.D._{600}$ of 0.8.

Following the cocultivation, the cotyledon explants were transferred to Petri dishes with selective medium consisting of MS20 supplemented with 4.56 μM zeatin, 67.3 μM vancomycin, 418.9 μM cefotaxime and 171.6 μM kanamycin sulphate, and cultured under the culture conditions described above. The explants were subcultured every 3 weeks onto fresh medium. Emerging shoots were dissected from the underlying callus and transferred to glass jars with selective medium without zeatin to form roots. The formation of roots in a medium containing kanamycin sulphate was regarded as an indication of the transgenic nature of the shoot in question. Truly transgenic regenerants were propagated *in vitro* by subculturing the

apical meristem and auxiliary buds into glass jars with fresh selective medium without zeatin.

EXAMPLE 10: COMPLEMENTATION ANALYSIS

Identification of cosmids with the *Mi* resistance gene by screening for resistance in roots of transformed plants

Roots of *in vitro* grown transformed R_0 plants have been subjected to the disease assay as described in Example 1. From each transformant two root explants have been assayed. In total 72 R_0 plants of 7 different cosmid transformations have been tested; 6 cosmids carrying tomato insert DNA and one cosmid, pJJ04541, is without tomato insert DNA. The results are shown in Table 1. Sixty three transgenic R_0 plants appeared susceptible, because galls had been formed on at least one of the two root cultures. Nine R_0 plants scored resistant, because no galls could be found on the root cultures. Seven resistant plants had been derived from transformation with cosmid Mi-11, while two resistant plants had been derived with cosmid Mi-18, that is overlapping for a great part with cosmid Mi-11. The cosmids Mi-11 and Mi-18 were used for further molecular analysis.

TABLE 1

Cosmid	R_0 plants	
	Resistant	Susceptible
Mi-32	0	8
Mi-30	0	11
Mi-11	7	4
Mi-18	2	8
Mi-01	0	10
Mi-14	0	15
pJJ04541	0	7

Molecular analysis of the transformed plants with a resistant phenotype

To demonstrate that the resistant phenotype of transgenic R_0 plants, which had been with the overlapping cosmids Mi-11 and Mi-18, is determined by the genomic insert present in the various cosmids, an AFLP analysis with the AFLP marker PM14 was performed. Selective restriction fragment amplification was performed with the primer combination identifying marker PM14 for the R_0 plants transformed with cosmids Mi-11 and Mi-18. The DNA fingerprints obtained showed the presence of the marker PM14 in the resistant plants indicating that the genomic insert present in cosmids Mi-11 and Mi-18 is also present in the R_0 plants and that the two identified overlapping cosmids Mi-11 and Mi-18 comprise the *Mi* resistance gene.

The inserts in cosmids Mi-11 and Mi-18 and the inserts in the adjacent cosmids Mi-32, Mi-30 on one side and cosmids Mi-01 and Mi-14 on the other side, were further characterized. The DNA region comprising the *Mi* resistance gene based on the overlap between the cosmids Mi-11 and Mi-18, was estimated at approximately 16-18 kb. Based on the susceptibility of the R_0 plants having the insert present in cosmid Mi-30, this region could be narrowed down to approximately 12 kb. A DNA segment comprising the *Mi* resistance gene, corresponding to the region flanked by the right ends of cosmids Mi-30 and Mi-11, was sequenced (see Figure 4).

EXAMPLE 11: NUCLEOTIDE SEQUENCE AND DEDUCED AMINO ACID SEQUENCE OF THE *Mi* RESISTANCE GENE FROM TOMATO

Subcloning of the overlapping DNA segment

To determine the sequence of the overlapping DNA segment in cosmids Mi-11 and Mi-18 containing the *Mi* resistance gene, a set of random subclones with a insert size of approximately 2 kb were generated. 7.5 µg of CsCl purified DNA of cosmids Mi-11 and Mi-18 was sheared for 10 seconds at 4°C at 15% probe power (in 40 µl 10mM Tris-acetate, 10mM Mg-acetate and 50mM K-acetate) using a Misonix (Misonix Inc., Farmingdale, NY, USA) sonicator (type XL2020) with a water filled cup horn (type 431A). Subsequently, the DNA was heated for 10 minutes at 60°C and

cooled to room temperature. The ends of the DNA fragments were repaired by adding 10µl of a repair mixture (10mM Tris-acetate, 10mM Mg-acetate, 50 mM K-acetate, 10U Klenow DNA polymerase, 10U T_4DNA polymerase and 2 mM of all 4 dNTP's) and followed by incubation for 30 minutes at 20°C. The sheared DNA was separated by electrophoresis on 1% Seakem GTG agarose gel (FMC Bio Products, Rockland, ME, USA). The fraction with a size of 1.8-2.2 kb was excised from the gel and subsequently the gel slice was digested with β-agarase I according to the protocol of the manufacturer (New England Biolabs Inc, Beverly, MA, USA) and the DNA was precipitated.

A modified pUC19 vector (designated pStuc) was used to clone the 1.8-2.2 kb fraction. In this vector the BamHI/SalI fragment of pUC19 was replaced by a DNA fragment containing a StuI, SpeI and SalI restriction site using two oligonucleotide primers and standard cloning techniques as described by Sambrook *et al.* (in: Molecular cloning: a laboratory manual, 1989, Cold Spring Harbor Laboratory Press). The 1.8-2.2 kb fraction was ligated at 16°C in the a StuI digested and dephosphorylated pStuc vector. The ligation mixture was subsequently transformed to Epicurian Coli XL2-Blue MRF' ultracompetent cells (Stratagene, La Jolla, CA, USA). Individual colonies were grown and stored in 384-wells microtiter plates (100 µl of LB medium containing 100 mg/l of carbenicillin).

To isolate clones representing the overlapping DNA region in cosmids Mi-11 and Mi-18 containing the *Mi* resistance gene, the 8.6 and 4.5 kb PstI fragment of cosmid clone Mi-18 (see Figure 4) as well as the AFLP marker PM14 were used as hybridization probes in colony hybridizations. Therefore, replicas of the 384-well grid of clones in microtiter plates were stamped onto Gene Screen Plus membrane filters (DuPont NEN, Boston, MA, USA) and allowed to grow into colonies on media.
Eighty four positive clones were used to isolate plasmid DNA using the alkaline lysis method as described by Ish-Horowicz *et al.* 1981, Nucl. Acids Res. 9, 2989-2997.

Sequence analysis

The ABI PRISM dye terminator cycle sequencing ready reaction kit was used to perform sequencing reactions in a Gene-Amp PCR system Model 9600 (Perkin-

Elmer, Foster City, CA. USA). Standard M13 forward and reverse primers were used. The reaction products were analyzed on 48 cm gels of an ABI Prism 377. The DNA sequence of 84 selected clones was determined using the standard forward and reverse sequencing primers. Sequence assembly and analysis was done with the 1994 version of the STADEN sequence analysis program (Dear and Staden, 1991, Nucl. Acids Res. 19, 3907-3911). A contiguous DNA sequence of approximately 9.9 kb nucleotides could be formed and is shown in Figure 5. A large open reading frame of 3621 nucleotides (ORF2) encoding a truncated polypeptide of 1206 amino acids (figure 7B) could be deduced.

EXAMPLE 12: ROOT-KNOT NEMATODE INFECTION: SOIL INOCULATION

Soil infected with the root-knot nematode *Meloidogyne incognita* had been prepared as follows: root systems of heavily infected tomato plants with a high number of galls (or root-knots), were cut into pieces and mixed through fresh soil.

Seeds were sown or small rooted plantlets were transferred into the infected soil. The plants were grown in a greenhouse at a temperature of 25°C. After 4 to 8 weeks, the plants were carefully pulled out of the soil and the roots were rinsed in water in order to remove the adhering soil. The level of infection was determined by counting the number of galls formed.

Plants were considered to be resistant when three galls or less were visible on the roots. Plants were considered susceptible when more than three galls were formed on the root system.

EXAMPLE 13: COMPLEMENTATION ANALYSIS

Identification of cosmids with the *Mi* resistance gene by screening for resistance in the selfed progenies of transformed plants

The primary regenerants (R_0 generation) of the transformation experiments were grown in the greenhouse for seed set. For each cosmid, ten to fifteen regenerants were grown and R_1 seeds were harvested. R_1 lines of at least seven R_0 plants of each cosmid were tested for resistance against *Meloidogyne incognita* in order to identify cosmids with the resistance gene. Twenty to 30 seedlings or plantlets of each R1 line were inoculated and evaluated as described in Example 12.

In total 63 R_1 lines of 7 different cosmid transformations have been tested; 6 cosmids carrying tomato insert DNA and one cosmid, pJJ04541, without tomato insert DNA. The results are shown in Table 2. Fifty-four transgenic R_0 plants appeared to be susceptible, because galls had been formed on the root systems of all tested R_1-plants. Nine R_0 plants are considered resistant, because at least half of the plants of each R_1 line had three or less galls. One R_1 line was completely resistant; six R_1 lines segregated in a ratio of about 3:1 (resistant to susceptible plantlets), and the progenies of two R_0 plants segregated 1:1. All the nine resistant R_0 plants had been derived from transformations with cosmid Mi-11.

Additional genetic evidence for the presence of the *Mi* resistance gene on cosmid Mi-11 was obtained in the next generation. Resistant R_1 plants were selfed. Fourteen of the resulting R_2 lines, which originated from four different R_0 plants, were tested for resistance against *M. incognita*. Twenty to thirty seedlings of each R_2 line were inoculated and evaluated as described in Example 12. The results are shown in Table 3. Five R_2 lines were completely resistant, indicating that the parental R_1 plants were homozygous for the *Mi* resistance gene. Eight R_2 lines segregated in a ratio of 3:1, indicating that their parental R_1 plants were heterozygous for the *Mi* resistance gene. One R_2 line was segregating in a ratio of about 1:1, and none of the tested lines appeared to be completely susceptible. These results prove that the selected R_1 plants, which are derived from several plants transformed with cosmid Mi-11, contain the functional *Mi* resistance gene.

TABLE 2

Cosmid	Number of R_1-lines of independent R_0 plants tested				
	Total	Segregation ratio R:S (resistant to susceptible)			
		1:0	3:1	1:1	0:1
Mi-32	7	0	0	0	7
Mi-30	9	0	0	0	9
Mi-11	9	1	6	2	0
Mi-18	8	0	0	0	8
Mi-01	10	0	0	0	10
Mi-14	9	0	0	0	9
pKK04541	11	0	0	0	11

TABLE 3

Cosmid	Number of R_2-lines of independent R_1 plants tested				
	Total	Segregation ratio R:S (resistant to susceptible)			
		1:0	3:1	1:1	0:1
Mi-11	14	5	8	1	0

EXAMPLE 14: POTATO APHID INFECTION ASSAY

Small tomato plants (4 weeks old) were inoculated with the potato aphid (*Macrosiphum euphorbiae*) by placing five to eight female aphids on the leaves. The plants were grown in the greenhouse at a temperature of 18° to 20°C. After one to two weeks the level of resistance was determined by counting the number of newly born aphids.

Plants were considered to be resistant when no living aphids were present on the stem or leaves. plants were susceptible when newly born aphids were present.

EXAMPLE 15: COMPLEMENTATION ANALYSIS

Identification of cosmids with the *Meu-1* resistance gene by screening for resistance in the selfed progenies of transformed plants.

A subset of the R_1 lines obtained in Example 13 was tested for resistance against *Macrosiphum euphorbiae* in order to identify cosmids with the *Meu-1* resistance gene. Ten to fifteen plantlets of each R_1 line were inoculated and evaluated as described in Example 14. In total 41 R_1 lines of 7 different cosmid transformations have been tested; 6 cosmids carrying tomato insert DNA and one cosmid, pJJ04541, without tomato insert DNA. The results are shown in Tabie 4. Thirty-six transgenic R_0 plants are considered susceptible, because dozens of aphids were proliferating on all or most plants of each R_1 line. Five R_0 plants are resistant, because at least half of the plants of each R_1 line were without living aphids. All these five resistant R_0 plants had been transformed with cosmid Mi-11.

The obtained results strongly indicate that the R_0 plants which are derived from transformations with cosmid Mi-11, contain a functional *Meu-1* resistance gene.

TABLE 4

Cosmid	Number of R_1-lines of independent R_0 plants tested				
	Total	Segregation ratio R:S (resistant to susceptible)			
		1:0	3:1	1:1	0:1
Mi-32	4	0	0	0	4
Mi-30	7	0	0	0	7
Mi-11	7	1	2	2	2
Mi-18	7	0	0	0	7
Mi-01	6	0	0	0	6
Mi-14	5	0	0	0	5
pJJ04541	5	0	0	0	5

Additional genetic evidence for the presence of the *Meu-1* resistance gene on cosmid Mi-11 was obtained in the next generation. Twenty-four R_2 lines that had been obtained from selfings of nematode resistant R_1 plants (see Example 13), which originated from nine different R_0 plants, were tested for resistance against *M. euphorbiae*. Eleven to fifteen seedlings of each R_2 line were inoculated and evaluated as described in Example 14. The results are shown in Table 5. One R_2 line segregated in a ratio of 3:1 and eight R_2 lines were segregating in a ratio of about 1:1. In these nine lines the potato aphid resistance phenotype is clearly visible. Five R_2 lines appeared to be completely susceptible. The remaining ten R_2 lines scored intermediate: they segregated in a ratio of about 1:3. These results indicate that several R_1 plants, which are resistant to *Meloidogyne incognita* and which are derived from R_0 plants transformed with cosmid Mi-11, have a functional *Meu-1* resistance gene.

In addition, eight R_1BC lines that were obtained from nematode resistant R_1 plants backcrossed with susceptible tomato line 52201 were tested for resistance against *M. euphorbiae*, in order to confirm inheritance of the introgressed *Meu-1* resistance gene. Twelve to fifteen seedlings of each R_1BC line were inoculated and evaluated as described in Example 14. The results are shown in Table 6.

The segregation ratios shown in Table 5 and Table 6 only serve to illustrate the inheritance of the resistance phenotype.

TABLE 5

Cosmid	Number of R_2-lines of independent R_1 plants tested					
	Total	Segregation ratio R:S (resistant and susceptible)				
		1:0	3:1	1:1	1:3	0:1
Mi-11	24	0	1	8	10	5

TABLE 6

Cosmid	Number of R_1BC-lines of independent R_1 plants tested					
	Total	1:0	3:1	1:1	1:3	0:1
Mi-11	8	0	1	5	2	0

EXAMPLE 16: TRANSCRIPT MAPPING

Transcript mapping studies were performed to map the 5' and 3' end of the *Mi*-resistance gene and to determine whether the *Mi* resistance gene contains any introns. The polymerase chain reaction to amplify parts of the transcripts from the *Mi* resistance gene was used for this purpose.

Total RNA from leaf tissue of the resistant tomato cultivar E22 was isolated according to the hot phenol method as described by Sambrook *et al* (in: Molecular cloning: a laboratory manual, 1989, Cold Spring Harbor Laboratory Press). Poly A+ RNA was isolated using biotinylated oligo(dT) bound to Dynabeads M-280 Streptavidin (DYNAL A.S., Oslo, Norway) according to the instructions of the manufacturer. A cDNA library was constructed using the Superscript Rnase H Reverse Transcriptase cDNA kit from Life technologies, Inc. Gaithersburg, MD, USA and the protocol supplied by the manufacturer.

5' and 3' RACE products were obtained using the Marathon cDNA amplification kit from Clontech (Paolo Alto, CA, USA). The primers used were designed based on the genomic Mi-sequence, and especially on the 5' end of the coding sequence of ORF2. Subsequently, the various 5' and 3'-RACE fragments were cloned into the TA cloning vector pCRII (Invitrogen Corporation, San Diego, CA, USA) and sequenced using standard protocols. The nucleotide sequences obtained were aligned with the 9.9 kb genomic sequence and two intron sequences could be deduced for the 5' end of the *Mi* resistance gene. One intron of 1306 nucleotides was located from nucleotide position 1936 to 3241 and the second one from nucleotide position 3305 to 3379 (Figure 5).

The largest Mi-transcript detected with the Marathon cDNA amplification kit maps at nucleotide position 1880. Hence, we conclude that the Mi transcriptional initiation site is positioned at or upstream of nucleotide 1880. The first ATG codon that could be detected within the 5' cDNA was located at nucleotide position 3263, 52 nucleotides upstream of the second intron, and a large open reading frame (ORF1) encoding a polypeptide of 1257 amino acids could be deduced and is shown in Figure 7A. As a result, this second intron is located between amino acid 14 and 15 of the *Mi*-resistance gene product.

EXAMPLE 17: PCR ANALYSIS OF MI-11 AND MI-18 TRANSFORMED PLANTS

Data obtained from complementation analysis in roots of transformed plants (Example 10) indicated that the *Mi* resistance gene was located on a DNA segment overlapping between cosmids Mi-11 and Mi-18, excluding the DNA segment corresponding to cosmid Mi-30, transformants of which were all susceptible. This region was estimated to be about 12 kb. However, in complementation analysis on the selved progenies of transformed plants, only cosmid Mi-11 transformed plants scored resistant (Examples 13 and 15). To address the question why Mi-18 transformed plants scored susceptible, a PCR analysis on the presence or absence of the putative Mi-ORF in transformed Mi-11 and Mi-18 plants was performed.

The following DNA samples have been analysed:

1. YAC clone 2/1256.
2-3. Cosmid Mi-11 in *E. coli* and in *A. tumefaciens*, respectively.
4-5. Cosmid Mi-18 in *E. coli* and in *A. tumefaciens*, respectively.
6. Tomato line E22 (resistant).
7. Tomato line 52201 (susceptible).
8-12. Five plants transformed with cosmid Mi-11.
13-17. Five plants transformed with cosmid Mi-18.

The DNA was digested with *PstI* and *PstI*-adaptors were ligated. Subsequently, a PCR analysis was performed with a primer identifying the *PstI* site and three additional selective nucleotides or marker PM14 and various PCR primers located upstream of PM14 using the enzym *rTh* polymerase (Gene Amp XL PCR kit; Perkin Elmer). The products generated varied in size from 443 to 6110 bp and encompass the complete PM14 upstream region of the putative Mi-ORF (see Figure 6).

It appeared that all templates generated PCR products of the expected size with the exception of the five plants transformed with cosmid Mi-18. Only the smallest PCR product (443 bp) was formed. These data indicate that almost the complete PM14 upstream region was not present in plants transformed with cosmid Mi-18. These deletions do not occur with cosmid Mi-18 present in *E. coli* or *A. tumefaciens* but occur only in transformed plants. Hence, we conclude that these deletions are responsible for the susceptible phenotype to *Meloidogyne incognita* and/or *Macrosiphum euphorbiae* of Mi-18 transformed plants.

EXAMPLE 18: NUCLEOTIDE SEQUENCE OF COSMID MI-11

The observation that only plants transformed with cosmid Mi-11 showed a resistant phenotype might indicate that additional open reading frames present on Mi-11 could be candidates to encode for resistance against nematodes and/or aphids. Therefore, the nucleotide sequence of the region upstream of the postulated ORF1 was determined to identify additional open reading frames.

A set of random subclones with an insert size of 2 kb were isolated using the 2.1, 4.7 and 2.9 kb PstI fragment of cosmid clone Mi-11 as hybridization probes in colony hybridization essentially as described in Example 11.

Fourty nine positive clones were used to determine the DNA sequence using the standard forward and reverse sequencing primers. Sequence assembly and analysis was performed as described in Example 11.

Three contiguous DNA stretches with sizes of 5618 bp (con25), 898 bp (con10) and 2495 bp (con62) could be deduced. The gaps between these DNA stretches

and the 9870 bp DNA sequence containing the putative Mi-ORF (Figure 6) was calculated using PCR and varied between 50-200 bp.

The three determined contigs (con25, con10 and con62) were analysed for the distribution of stop codons in all six possible frames. No significant ORF frames with a size of or superior to 120 amino acids could be postulated. In addition, no DNA homology with the putative ORF1 was detected. Hence, the only significant ORF present on cosmid Mi-11 was ORF1 as described in Figure 5. Based on these results, it can be concluded that the polynucleotide encoded by ORF1 confers resistance to nematodes as well as to aphids and, hence, that the *Mi*-resistance gene and the *Meu-1* resistance gene are referring to the same coding sequence as depicted in figure 5.

EXAMPLE 19: TRANSFORMATION OF TOBACCO AND COMPLEMENTATION ANALYSES

Transformation of tobacco

The tobacco cultivar Petit Havana, type SR1, was transformed with cosmid Mi-11 or the cosmid vector pJJ04541 using the protocol as described by Horsch *et al.* (Science **227**, 1229-1231, 1985).

Complementation analysis: screening for nematode resistance in root cultures of transformed tobacco plants

Roots of *in vitro* grown transformed R_0 plants of tobacco have been subjected to the disease assay as described in Example 1. From each of the 31 transformants two or more root explants have been assayed. In addition, all 17 Mi-11 transformants have been analyzed by PCR for the presence of the putative Mi ORF1 by screening for an internal fragment with a size of 823 base pairs (ranging from nucleotide position 4824 to 5646, see Figure 5). Simple PCR primers for the fragment were deduced from the sequence as shown in Figure 5. The primers used have the following sequences:

primer S21: 5'-CCAAGGACAGAGGTCTAATCG-3'

primer S22: 5'-TTGAGGTGATGTGGTAAATGG-3'

Primer S21 targets the sequence from nucleotide position 4824 to 4844 and primer S22 targets the sequence from nucleotide position 5626 to 5646 (see Figure 5).

The results of the *in vitro* disease assay and of the PCR analysis (presence "+" or absence "-" of the internal PCR fragment) are shown in Table 7. "Mi-11" represents transformed plants comprising the putative Mi ORF1 and "Mi-11Δ" represents those transformed plants having a deletion in the putative Mi ORF1, as determined by the PCR analysis (described above). Twenty-nine R_0 transformants were susceptible, because galls had been formed on at least one of the tested root cultures. Generally, the rate of gall formation on tobacco roots is slightly lower than on susceptible tomato roots. Two R_0 plants scored resistant to *Meloidogyne incognita*, because no galls could be found on the root cultures. Both resistant plants were transformed with cosmid Mi-11 comprising the internal PCR fragment indicating the presence of the *Mi* resistance gene.

TABLE 7

Genotype	PCR fragment	R_0 plants	
		Resistant	Susceptible
Mi-11	+	2	7
Mi-11Δ	-	0	8
pJJ04541	-	0	14

Complementation analysis: screening for aphid resistance in cuttings of transformed tobacco plants

Rooted cuttings of transformed R_0 plants of tobacco were inoculated and evaluated as described in Example 14. From each of the 23 transformants two or three cuttings have been assayed for resistance against *Macrosiphum euphorbiae*. The results of the infection assay and the PCR analysis (as described above) are shown in Table 8. Twenty-one R_0 plants are considered susceptible, because several living aphids were counted on at least one of the tested cuttings. In general, the level of proliferation of the aphids on tobacco is low compared with the proliferation on susceptible tomato plants. Two R_0 plants scored resistant, because all cuttings of these plants were without living aphids. The aphid resistant plants were transformed with cosmid Mi-11, comprising the *Mi* resistance gene, as indicated by the presence of the internal PCR fragment.

TABLE 8

Genotype	PCR fragment	R_0 plants	
		Resistant	Susceptible
Mi-11	+	2	3
Mi-11Δ	-	0	6
pJJ04541	-	0	12

EXAMPLE 20 TRANSFORMATION OF POTATO AND COMPLEMENTATION ANALYSES

Transformation of potato

The potato variety Diamant (Cebeco Zaden B.V., Vlijmen, The Netherlands) was used for transformation. Internode explants of *in vitro* grown plants were transformed with cosmid Mi-11 or the cosmid vector pJJ04541 using the protocol as described by Ooms *et al.* (Theor. Appl. Genet. **73**, 744-750).

Complementation analysis: screening for nematode resistance in root cultures of transformed plants

Roots of *in vitro* grown transformed R_0 plants of potato have been subjected to the disease assay as described in Example 1. From each of the 31 transformants at least two root explants have been assayed. In addition, all 26 Mi-11 transformants have been analyzed by PCR using primers S21 and S22 as described in Example 19. The results of the *in vitro* disease assay and of the PCR analysis (presence "+" or absence "-" of the internal PCR fragment) are shown in Table 9. "Mi-11" represents transformed plants comprising the putative Mi ORF1 and "Mi-11Δ" represents those transformed plants having a deletion in the putative Mi ORF1, as determined by the PCR analysis (described above). Twenty-eight R_0 transformants were susceptible, because galls had been formed on at least one of the root cultures. Generally, the rate of gall formation on potato roots is lower than on susceptible tomato roots. Three R_0 plants scored resistant to *Meloidogyne incognita*, because no galls could be found on the root cultures. All these resistant plants were transformed with cosmid Mi-11 comprising the internal PCR fragment indicating the presence of the *Mi* resistance gene.

TABLE 9

Genotype	PCR fragment	R_0 plants	
		Resistant	Susceptible
Mi-11	+	3	17
Mi-11Δ	-	0	6
pJJ04541	-	0	5

Complementation analysis: screening for nematode resistance in cuttings of transformed plants

Rooted cuttings of Mi-11 transformed R_0 plants of potato have been subjected to the disease assay as described in Example 12. From each of the 19 transformants one to three cuttings have been assayed for resistance against *Meloidogyne incognita*. The results are shown in Table 10. In addition, 36 rooted cuttings of non-transformed potato plants (variety Diamant) were assayed (as susceptible controls) and were all susceptible. One R_0 plant scored resistant to *Meloidogyne incognita*, because no galls could be found on the root system.

TABLE 10

Genotype	PCR fragment	R_0 plants	
		Resistant	Susceptible
Mi-11	+	1	12
Mi-11Δ	-	0	6
Non-transf. control	-	0	1

CLAIMS

1. A nucleic acid whose DNA sequence is the DNA of figure 5 or part thereof or a DNA sequence homologous to the DNA sequence of figure 5.

2. The nucleic acid of claim 1 which is capable, when transferred to a host plant, which is susceptible to a plant pathogen, of rendering said host plant resistant to said plant pathogen.

3. A nucleic acid sequence which is a cDNA corresponding to a nucleic acid whose DNA sequence is at least part of the DNA sequence provided in figure 5.

4. The nucleic acid of claim 1 or 3, which, when transferred to a host plant is capable of rendering it resistant to nematodes.

5. The nucleic acid of claim 1 or 3, which, when transferred to a host plant is capable of rendering it resistant to aphids.

6. The nucleic acid of claim 1 or 3, which, when transferred to a host plant is capable of rendering it resistant to nematodes and aphids.

7. A nucleic acid wherein said DNA sequence corresponds to a sequence starting at nucleotide 3263 and ending at nucleotide 7111 of the sequence of figure 5 or any DNA sequence homologous thereto.

8. A nucleic acid wherein said DNA sequence corresponds to a promoter sequence located 5' upstream of nucleotide 3263 or any DNA sequence homologous thereto.

9. A nucleic acid of claim 1 or 3 wherein said DNA sequence corresponds to at least part of the genomic insert present in cosmid Mi-11, or any DNA sequence homologous thereto.

10. A recombinant DNA construct comprising a nucleic acid according to any of claims 1-9.

11. A recombinant DNA construct of claim 10 in which said nucleic acid is under control of a promoter which is functional in a plant cell, said promoter being either endogenous or exogenous to said plant cell, and effective to control the transcription of said DNA sequence in such plant cells.

12. A recombinant DNA construct of claim 11 in which said promoter corresponds to a promoter sequence located 5' upstream of nucleotide 3263 as provided in figure 5, or any DNA sequence homologous thereto.

13. A vector suitable for transforming plant cells comprising a DNA construct according to any of claims 10-12.

14. Plasmid pKGMi-11 as deposited under number CBS 822.96.

15. Plasmid pKGMi-18 as deposited under number CBS 821.96.

16. Bacterial cells comprising a vector or plasmid according to any of claims 13-15.

17. Recombinant plant genome comprising, incorporated thereinto, a DNA construct according to any of claims 10-12.

18. Plant cells comprising a DNA construct according to any of claims 10-12.

19. Plant comprising plant cells according to claim 18.

20. Plant according to claim 19 which has a reduced susceptibility to nematodes.

21. Plant according to claim 20 wherein said nematode is a root-knot nematode, especially *Meloidogyne incognita*.

22. Plant according to claim 19 which has a reduced susceptibility to aphids, especially *Macrosiphum euphorbiae*.

23. Seed comprising a DNA construct according to any of claims 10-12.

24. The recombinant plant genome of claim 17, in a plant cellular environment.

25. Process for obtaining plants having reduced susceptibility to a pathogen, comprising the following steps:

i) inserting into the genome of a plant cell a DNA construct according to any of claims 10-12,

ii) obtaining transformed plant cells,

iii) regenerating from said transformed plant cells genetically transformed plants, and

iv) optionally, propagating said plants.

26. Process according to claim 25 wherein said pathogen is a nematode, and preferably a root-knot nematode, especially *Meloidogyne incognita*.

27. Process according to claim 25 wherein said pathogen is an aphid, and preferably *Macrosiphum euphorbiae*.

28. Process for protecting plants in cultivation against pathogen infection, which comprises:

i) providing the genome of plants with a DNA construct according to any of claims 10-12, and

ii) growing said plants.

29. Process for isolating a nucleic acid according to claim 1-6, comprising the following steps:

i) screening a genomic or cDNA library of a plant with a DNA sequence according to claim 1-9,

ii) identifying positive clones which hybridize to said DNA sequence,

iii) isolating said positive clones.

30. The process of claim 29 wherein said library originates from a first plant and the DNA sequence belongs to a second plant.

31. Process of selective restriction fragment amplification for identifying a nucleic acid according to claim 1-9 using primer combinations identifying at least one of the AFLP markers PM02 to PM29 as depicted in Table 3.

32. The process of claim 31 wherein said primer combination identifies AFLP marker PM14.

33. An oligonucleotide comprising a DNA sequence which corresponds to at least part of the nucleic acid according to claims 1-9.

34. The oligonucleotide of claim 33, which is of a size sufficient to hybridize selectively to the DNA sequence of any of claims 1 to 9 under stringent hybridization conditions.

35. An oligonucleotide according to claim 34 wherein said DNA sequence corresponds to the sequence starting at nucleotide 6921 and ending at nucleotide 7034.

36. An oligonucleotide according to claim 35 wherein said DNA sequence is located at the 3'end, and preferably corresponds to the sequence 5'TGCAGGA-3', which can prime the synthesis of DNA.

37. An oligonucleotide according to claim 35 wherein said DNA sequence is located at the 3'end, and preferably corresponds to the sequence 5'-TAATCT-3' which can prime the synthesis of DNA.

38. A primer combination comprising a first oligonucleotide according to claim 36 and a second oligonucleotide according to claim 37.

39. Diagnostic kit comprising at least one oligonucleotide according to any of claims 33-37.

40. Diagnostic kit comprising a primer combination according to claim 38.

41. Process for detecting the presence or absence of a DNA sequence according to claim 1-9, particularly in a plant DNA using a diagnostic kit according to claim 39 or 40.

42. A polypeptide which is the expression product of a nucleic acid of recombinant DNA according to anyone of claims 1 to 13.

43. A polypeptide having an amino acid sequence having the sequence provided in figure 7A or coded by the corresponding homologous sequence according to anyone of claims 1 to 3.

44. A RNA having a ribonucleic acid sequence of a transcript of part or all of the DNA sequence of anyone of claims 1 to 3.

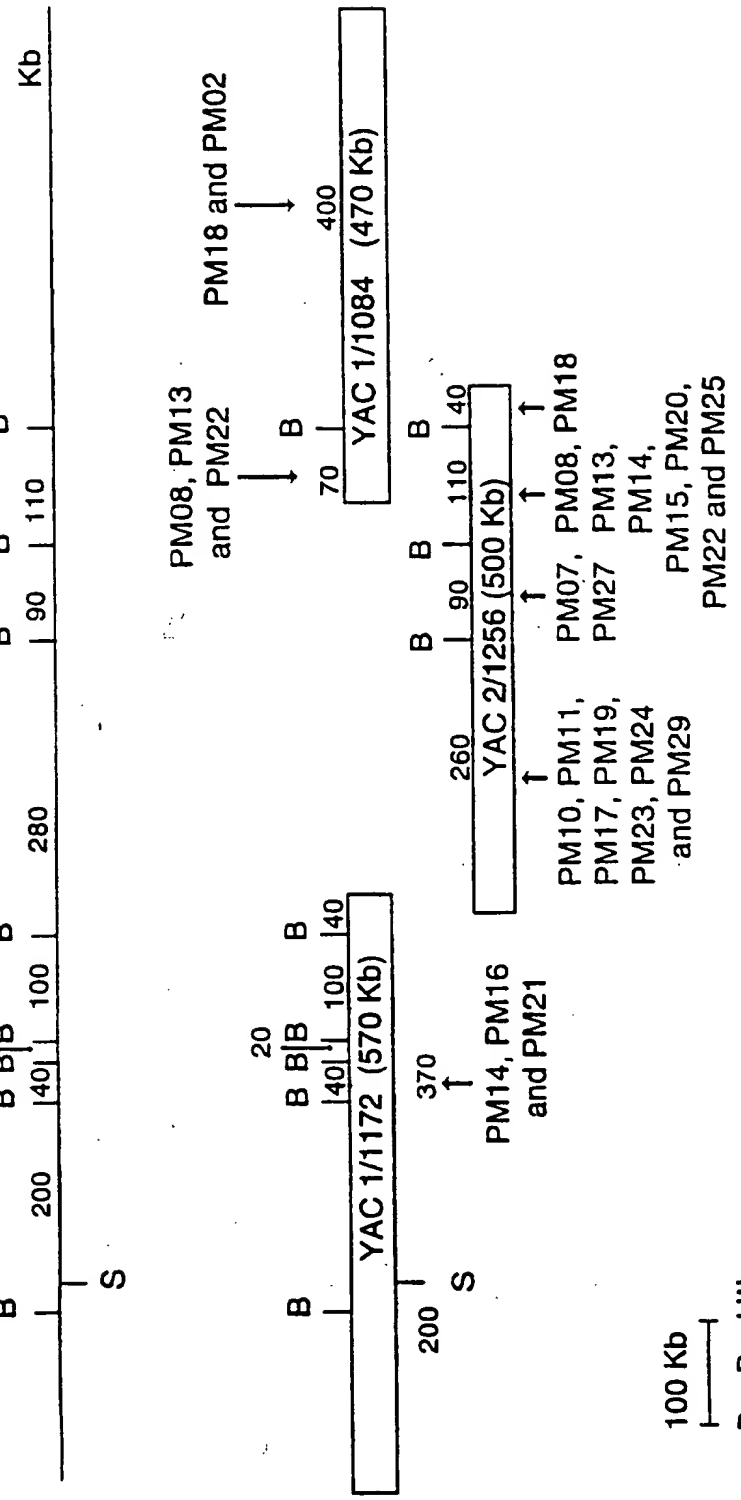
Kb
B
S
20
B B B
40
100
200
B
B
B
280
B
B
B
90
110
PM08, PM13
and PM22
PM18 and PM02
B
70
400
YAC 1/1084 (470 Kb)
B
B
B
90
110
40
260
YAC 2/1256 (500 Kb)
PM10, PM11,
PM17, PM19,
PM23, PM24
and PM29
PM07, PM08, PM18
PM27 PM13,
PM14,
PM15, PM20,
PM22 and PM25
20
B B B
40
100
200
B
B
B
40
370
YAC 1/1172 (570 Kb)
PM14, PM16
and PM21
S
100 Kb
B = BssHII
S = SfiI

FIGURE 1

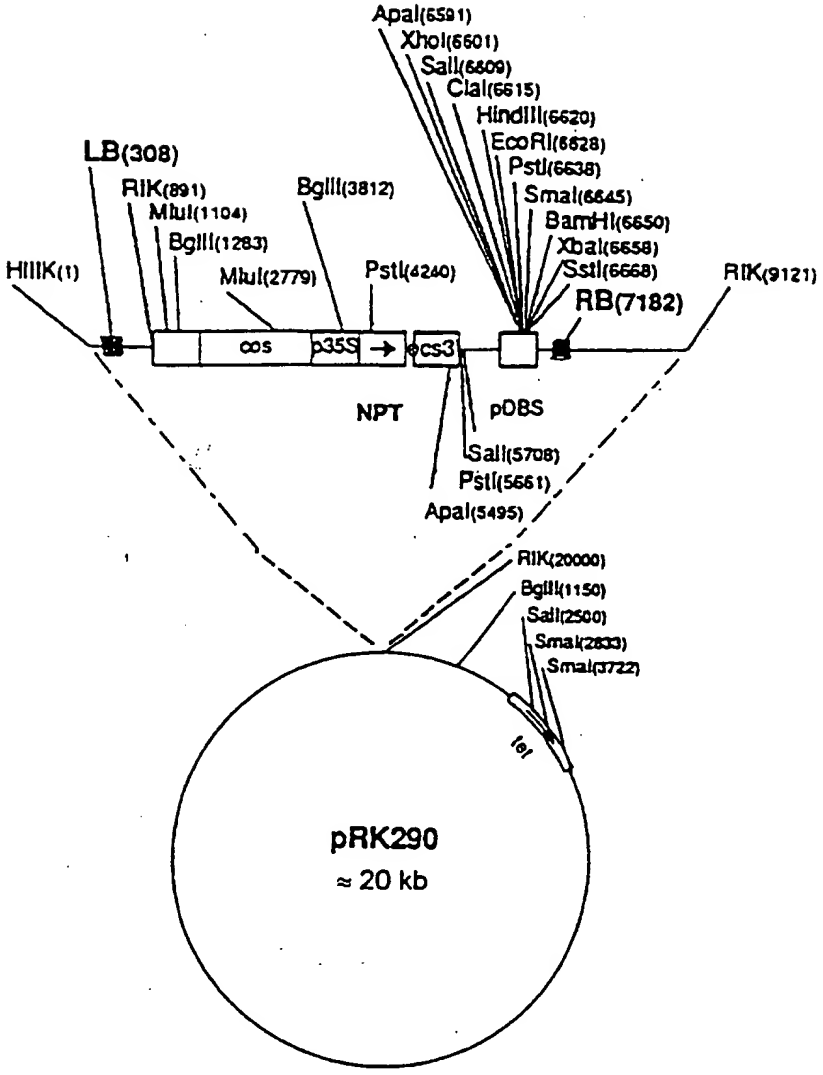
ApaI(6591)
XhoI(6601)
SalI(6609)
ClaI(6615)
HindIII(6620)
EcoRI(6628)
PstI(6638)
SmaI(6645)
BamHI(6650)
XbaI(6658)
SstI(6668)
RB(7182)
LB(308)
RIK(891)
MluI(1104)
BglII(1283)
MluI(2779)
BglII(3812)
PstI(4240)
HIIIK(1)
RIK(9121)
cos
p35S
cs3
NPT
pOBS
SalI(5708)
PstI(5661)
ApaI(5495)
RIK(20000)
BglII(1150)
SalI(2500)
SmaI(2833)
SmaI(3722)
lel
pRK290
≈ 20 kb

FIGURE 2

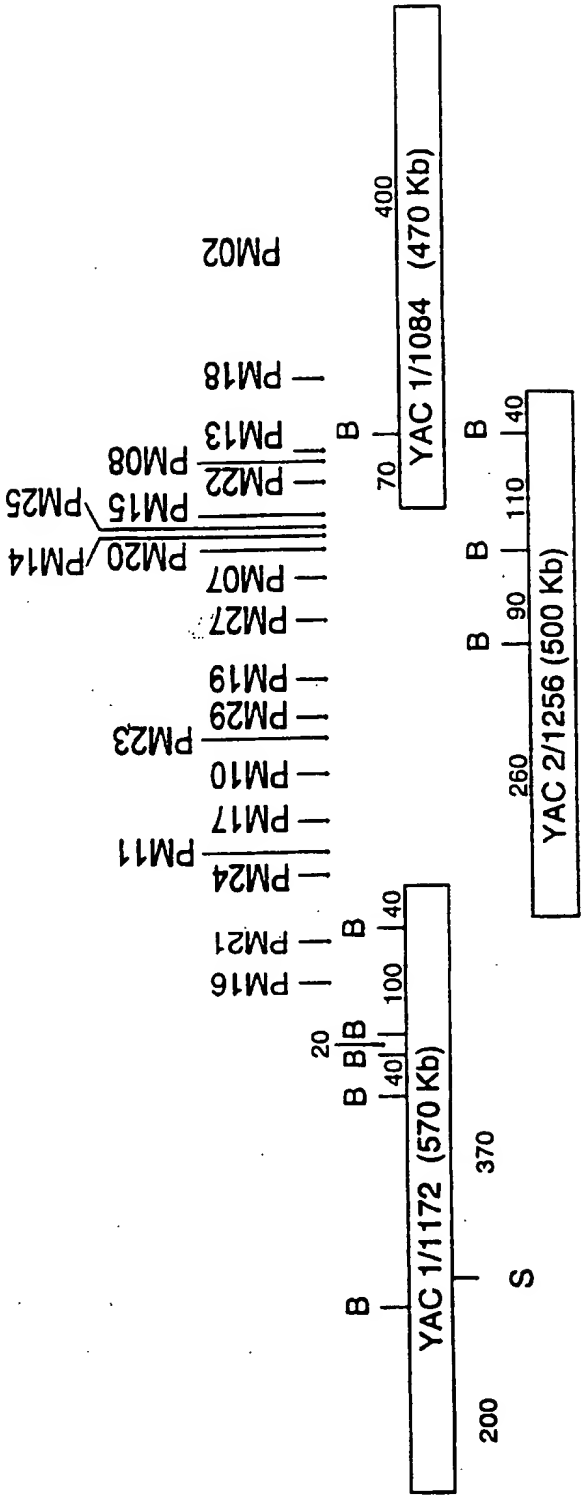
YAC 1/1172 (570 Kb)
YAC 1/1084 (470 Kb)
YAC 2/1256 (500 Kb)
B
S
PM02
PM18
PM13
PM08
PM22
PM15
PM25
PM20
PM14
PM07
PM27
PM19
PM29
PM23
PM10
PM17
PM11
PM24
PM21
PM16
100 Kb
B = BssHII
S = SfiI

FIGURE 3A

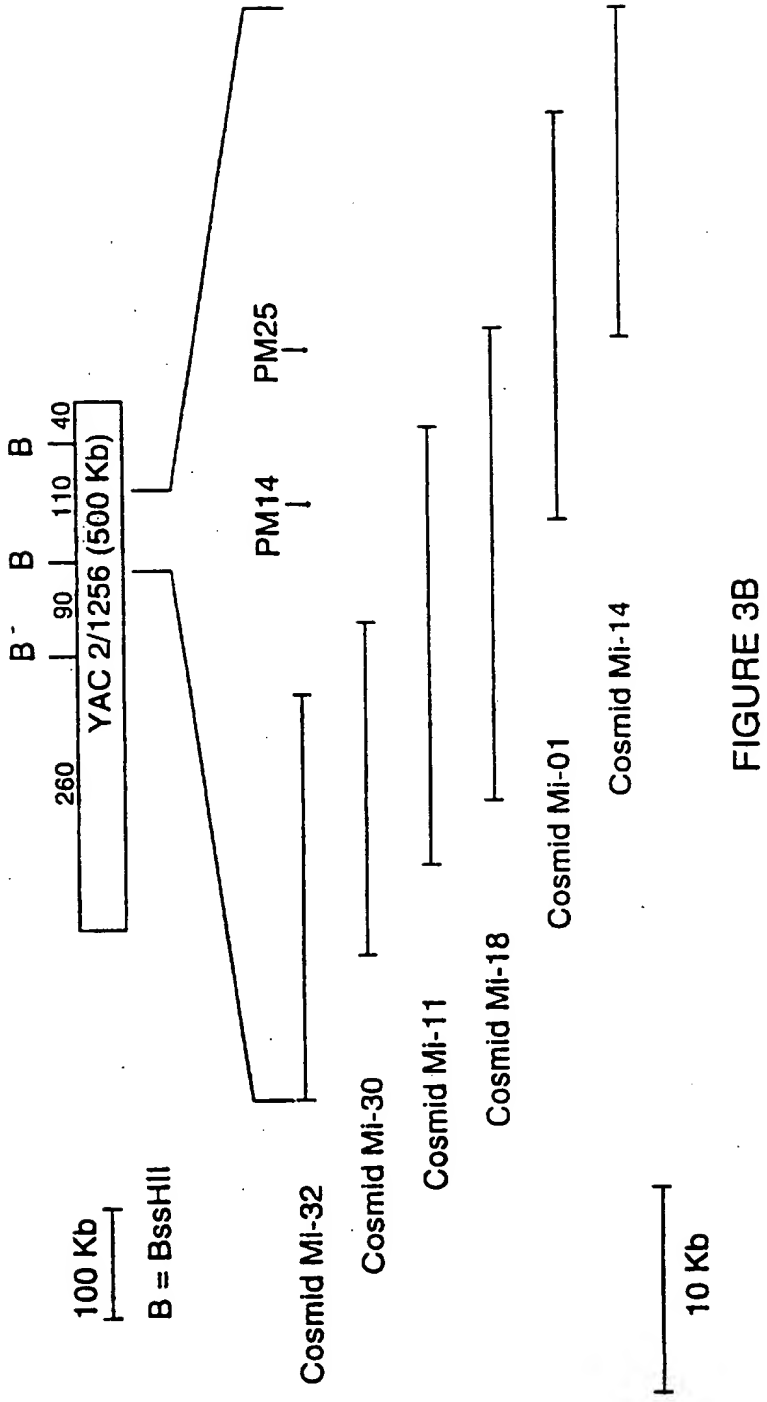
B
B
B
40
110
90
260
YAC 2/1256 (500 Kb)
PM25
PM14
Cosmid Mi-32
Cosmid Mi-30
Cosmid Mi-11
Cosmid Mi-18
Cosmid Mi-01
Cosmid Mi-14
100 Kb
B = BssHII
10 Kb

FIGURE 3B

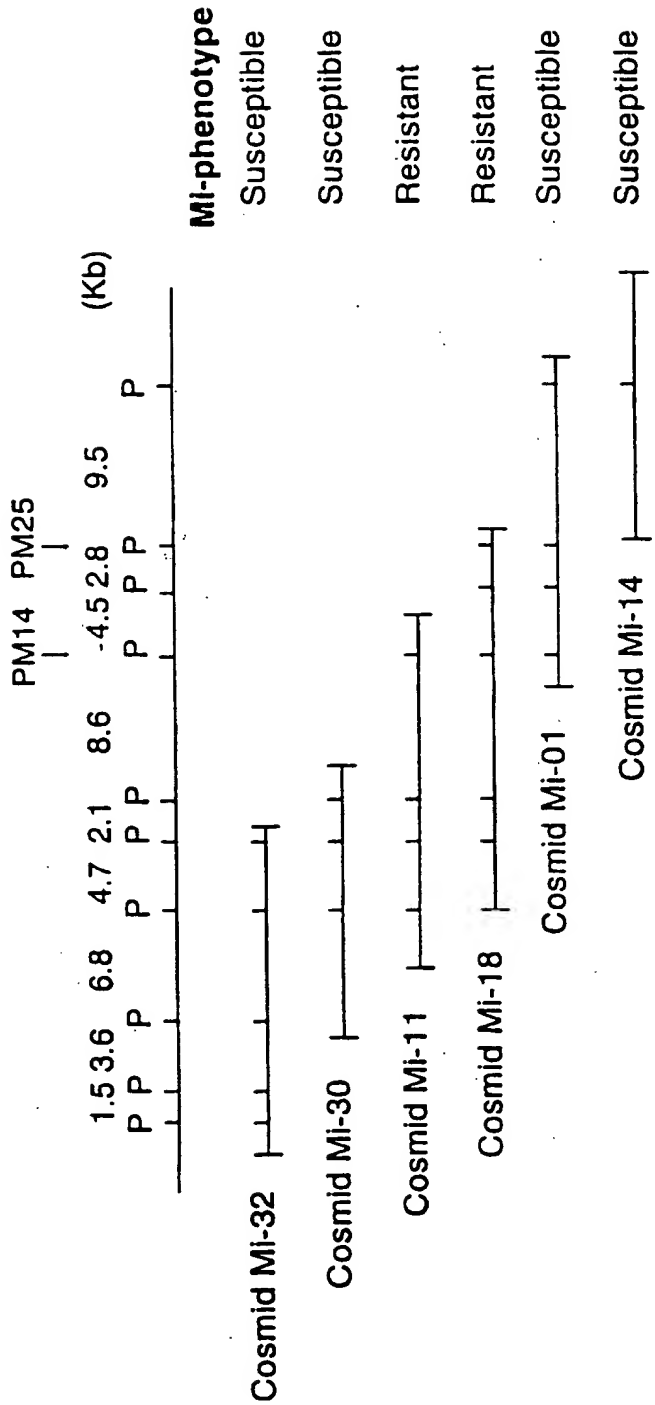
Mi-phenotype
Susceptible
Susceptible
Resistant
Resistant
Susceptible
Susceptible
(Kb)
PM14 PM25
1.5 3.6 6.8 4.7 2.1 8.6 -4.5 2.8 9.5
P P P P P P P P P
Cosmid Mi-32
Cosmid Mi-30
Cosmid Mi-11
Cosmid Mi-18
Cosmid Mi-01
Cosmid Mi-14
Sequence
P = Pst I
10 Kb

FIGURE 4

```
5'-TTTTCCTCTTCATATAACTTTTTCCTTAACCCCTCTCATGAATAATATAATTGATGTGGA     60
   TAAAGTATTATCCTTTATGATAAATAACGAAATTTAATAATTTAAAGGGTGCAAATCTAT    120
   AAAATGGAGACGCACATTGATAATGTCCTCTTGATTATTATTAAAGAATTACTCTAGCTT    180
   CACAAATTTAAATTCATTAATGCTTAATTACATGATAAAAACTTTAGTTGTTCTTTTTAC    240
   ATGGTTTGCTAACTTTAATTTTTTTTCTTCATATTCTTCATTTGTTTATTATTATTTTCT    300
   AATTACTTATTTAACTTTTATACTCTTAATATTCATAACTCTCATCTTTTCATATTCATA    360
   ACCTCCAAATATTTAAACTAAAACTTTAAGATATCTTTTGATATTTGTTCAATAATAAAT    420
   TCAACTTCTTTATCTTATGAAACCCCTACCAAGATTATTAGGCTATTATTTTTTATTCTA    480
   TAGTAAAAACAAATGATGAAGATTCTTGAATTTTATAGGATATGAAAGAAGTCGATAAAA    540
   TCTCAGAGAGTTATGTACTAATTTTGTACTTATTTTTTCATCTATATATACATAAATCTT    600
   ATAAGAATAATGTCTATATTGTATTTTTTTCTTAAATATTATGTTTCTTTTTAATTTTTT    660
   TTCACTCTGTTAGACTTCTTAATTTAGTTTTCTATGAATGTTTTATTGCCGTAAGTCTTT    720
   GAATTTTGTAATTGTTACATTTTATTATTCATTACGATTTACATATATATTTCCATGAGA    780
   TTTGGTCATTCTAACGTATCTATAAAAATTCACATGAAACACACGTGTGAAGCGCATCCT    840
   CAGAAAAACTAGTGTATATATATATATATATATATATATATATATATATATATATATATA    900
   TATATATATATATATATATATATATATTATTCTTATTAAAAAAGAATGTCCTTATTTCAT    960
   TTTTAATCTGGTTAAAAAAGAATAATCTCTTTCCTTTTTTGACAATATTTTAACTTTAAC   1020
   TTTCCACGTAACATGTTTAAGACAACAAAATTAAATGACATTTTAATCTTGTAACATAGA   1080
   AAAGTAACATATGATAATTGTCGTTGTCCCTAAACATGATAGATGTATAATTCAAAAGTC   1140
   AATGAATTGTATTTTAGTATTATATTATGAATGAACAAACTGTCAAGATGTGTATATATA   1200
   TATATATTTTATTCTTGTTAATTTGGCCTTTCAAGTAATTAATTCATTGTTAGGCAGTTG   1260
   AATTAATAATCTCTTTTAGGAATCTTCCCATGTGAATAACAAGACTTATAATAATAATAA   1320
   TAAAGTCCAGATCTTGTTTCAATTGGATCATTTGGCAAACAATTACTCTGTTTCTGAAAC   1380
   AAGGAATAGGGCTTCTAATATTGTAGGGGATTTTTTTTTCTTCATTAATTTATACTTATG   1440
   ATATTAATTATTGTTTTTGAGTACATATTTTAAACTCTGTTGTTTATTTTTCTGCAAAGT   1500
   TTCTCCGGTTATATTGAACATATACACATATAGTACATATATTTATTGTAAAAAAAATAA   1560
   TTATTATACTCCATTTCAAGAAATTATGTTTTGATATTATATATTAAATTCTATAATGTG   1620
   GAAATTGTCAATGTCTACAATGTGTTTGATGAAATGACAACCACTTGTTTTTATCTGCAA   1680
   CAGTATAAAAATTGGCTTTGCTTCTTTTAGATTAATATAATATTTTACAGGTCACATATT   1740
   ATATTTATATTGTGAAAGACAAGAGATATTGATTAAAAAAAGACTTATGGGTTTGTATTT   1800
   TAATATTTCATTCTTCTTCATTACTAAAAGACTTGTATCGTATATTTCAACTACTACACT   1860
   TGTTTTCTTATCCAATAGCTTCAACATTATTTCTCAAACAAAGGGTTCTCTAGCTAAACT   1920
   TCAGCCTGTGTAAAGGTAACATCTTCTTTATTCACAGCATAATAACAATGAATTTGGTCG   1980
   ATGTTTGAAGTAAGCTTGAAATTTTCTCTTTCTAAGTTTGTTTGATCCATTTAGATTCTT   2040
   TTAAATACTTTTGGTATTTAAAGGACTTGTGAAGTCAATGAATTGTATTTTAGTAATCTT   2100
   GCAATTCTAGATCTAGCTATTTGTTGTTCTCCTTTCAACCAAACTACTTCTTCAATTTGT   2160
   CTAACAAAAATATGTCAAAAAGGTATGAACATGCTTAATCGGAGATCTTTATTGATTCTA   2220
   CTTCAGCTACTCTAAAAAAAAAATCTTTTTTCCATTAAGCCCAAGTCGAGATAGGAGAAAA   2280
   ATATTATTAGAGAGATTATTAATTTAATGACATTTTACTCTAGTTTTTTATCAAAATAAG   2340
   GGAATAATATCCTGTTATTTAACTACCTTTTAAGCATTATGGGTGGAAAGTAGAAAGAAG   2400
   AAACATAACAGAACAGACAGTAAGTTATGCTTTAATGAGTAGATCTGTATAGGATTACAT   2460
   ATTTGTTTGACTTTTCGGTGTTTCGATTAGAAAACTTACAAGTTTTTAATACATGTATCA   2520
   TTTGTTGATTTGTCCGTTTGGCACGTCATCTGTGGTTACAAGTCACATATGAAGTATGTC   2580
   CACGAGACACACCGAATGTCAAGTATAGATTTCTACTTGATCATACACAACTTTATCTGA   2640
   GGTTGATGCCAAATTTAAATGACTACCTAAAGCTGATATTTTAAACATTAATCTTGTACA   2700
   CGAAAACATTATTCCTATTACTGTTTTCTTTACCTTTACCTTATAGACTTTTTTGGCAGA   2760
   AAAAAGTTAGACAGATACATTTGATGATGTTTACCATTCTCATTCTCTCTTTATTTTATT   2820
   TTCTTTACATTCACACGCACAATAATTTTCTTGTAGGTTCCTTATATGCCATATGCACAT   2880
   AGACGAATCTAGGATTTGATATTTACAAGTTTCTATGTCGACGTCATATTAATATCAATA   2940
   ATAATTAGATTGACAATCACATATTTATAATATTAAGTCGATAACTTTCTTCTTTGTATA   3000
   GGTTGGAAAAGTAATGGTAAACGAGCAGGACTCCTTTTTCTTTTTTTTTGTAAATAATTAA   3060
   CAGTTGTGAGATTTTATGTTTGTGACTTCATGTCATAAACATTTTGATGTGTGATTAAGA   3120
```

FIGURE 5a

```
TTGACATTTCCAATTGTGCGAGTCTAAAATTACTATATGTGAAAATAGTGATATTATTGA  3180
TTATTCGTATTTTTTCATCTTCTTTCTCCTGTTAAAGTTTTATCTACTTTTTATTCATCA  3240
GGTCTTGAGAAAAAGTAGAATCATGGAAAAACGAAAAGATATTGAAGAAGCAAACAACTC  3300
                        M  E  K  R  K  D  I  E  E  A  N  N  S    13
ATTGGTATGTTATTTTATAGAGTAAACTGTAAAGTATTGAATTATAGATATGTGGCTTTA  3360
 L                                                              14
AAATGTATTATTTTGGCAGGTGTTATTTTCTGCTCTTAGCAAGGACATTGCCAATGTTCT  3420
                     V  L  F  S  A  L  S  K  D  I  A  N  V  L    28
AATTTTCCTAGAGAATGAGGAAAATCAAAAAGCTCTTGACAAAGATCAAGTTGAAAAGCT  3480
  I  F  L  E  N  E  E  N  Q  K  A  L  D  K  D  Q  V  E  K  L    48
AAAATTGAAAATGGCATTTATTTGTACATATGTTCAGCTTTCTTATTCCGATTTTGAGCA  3540
  K  L  K  M  A  F  I  C  T  Y  V  Q  L  S  Y  S  D  F  E  Q    68
GTTTGAAGATATAATGACTAGAAATAGACAAGAGGTTGAGAATCTGCTTCAATCACTTTT  3600
  F  E  D  I  M  T  R  N  R  Q  E  V  E  N  L  L  Q  S  L  L    88
GGATGATGATGTCCTTACTAGCCTCACCAGTAATATGGATGACTGTATCAGCTTGTATCA  3660
  D  D  D  V  L  T  S  L  T  S  N  M  D  D  C  I  S  L  Y  H   108
TCGTTCTTATAAATCAGATGCCATCATGATGGATGAGCAATTGGACTTCCTCCTCTTGAA  3720
  R  S  Y  K  S  D  A  I  M  M  D  E  Q  L  D  F  L  L  L  N   128
TCTGTATCATCTATCCAAGCATCACGCTGAAAAGATATTTCCTGGAGTGACTCAATATGA  3780
  L  Y  H  L  S  K  H  H  A  E  K  I  F  P  G  V  T  Q  Y  E   148
AGTTCTTCAGAATGTATGTGGCAACATAAGAGATTTCCATGGGTTGATACTGAATGGTTG  3840
  V  L  Q  N  V  C  G  N  I  R  D  F  H  G  L  I  L  N  G  C   168
CATTAAGCATGAGATGGTTGAGAATGTCTTACCTCTGTTTCAACTCATGGCTGAAAGAGT  3900
  I  K  H  E  M  V  E  N  V  L  P  L  F  Q  L  M  A  E  R  V   188
AGGACACTTCCTTTGGGAGGATCAGACTGATGAAGACTCTCGGCTCTCCGAGCTAGATGA  3960
  G  H  F  L  W  E  D  Q  T  D  E  D  S  R  L  S  E  L  D  E   208
GGATGAACACAATGATAGAGACTCTCGACTCTTCCAGCTAACACATCTACTCTTGAAGAT  4020
  D  E  H  N  D  R  D  S  R  L  F  Q  L  T  H  L  L  L  K  I   228
TGTTCCAACTGAACTGGAGGTTATGCACATATGTTATACAAATTTGAAAGCTTCAACTTC  4080
  V  P  T  E  L  E  V  M  H  I  C  Y  T  N  L  K  A  S  T  S   248
AGCAGAAGTTGGACGCTTCATTAAGAAGCTCCTGGAAACCTCACCGGATATTCTCAGAGA  4140
  A  E  V  G  R  F  I  K  K  L  L  E  T  S  P  D  I  L  R  E   268
ATATATCATTCAACTACAAGAGCATATGTTAACTGTTATTCCCCCTAGCACTTTAGGGGC  4200
  Y  I  I  Q  L  Q  E  H  M  L  T  V  I  P  P  S  T  L  G  A   288
TCGAAACATTCATGTCATGATGGAATTCCTATTACTTATTCTTTCTGATATGCCCAAGGA  4260
  R  N  I  H  V  M  M  E  F  L  L  L  I  L  S  D  M  P  K  D   308
CTTTATTCATCATGACAAACTTTTTGATCTCTTGGCTCATGTTGGAACACTTACCAGGGA  4320
  F  I  H  H  D  K  L  F  D  L  L  A  H  V  G  T  L  T  R  E   328
GGTATCGACTCTTGTACGTGACTTGGAAGAGAAATTAAGGAATAAAGAGGGTAATAACCA  4380
  V  S  T  L  V  R  D  L  E  E  K  L  R  N  K  E  G  N  N  Q   348
AACAAATTGTGCAACCCTAGACTTGCTGGAAAATATTGAACTCCTCAAGAAAGATCTCAA  4440
  T  N  C  A  T  L  D  L  L  E  N  I  E  L  L  K  K  D  L  K-  368
ACATGTTTATCTGAAAGCCCCAAATTCATCTCAATGTTGCTTCCCCATGAGTGATGGACC  4500
  H  V  Y  L  K  A  P  N  S  S  Q  C  C  F  P  M  S  D  G  P   388
ACTCTTCATGCATCTTCTACACATGCACTTAAATGATTTGCTAGATTCTAATGCTTATTC  4560
  L  F  M  H  L  L  H  M  H  L  N  D  L  L  D  S  N  A  Y  S   408
AATTTCTTTGATAAAGGAAGAAATCGAGTTGGTGAGTCAAGAACTGGAATTCATAAGATC  4620
  I  S  L  I  K  E  E  I  E  L  V  S  Q  E  L  E  F  I  R  S   428
ATTCTTTGGGGATGCTGCTGAGCAAGGATTGTATAAAGATATCTGGGCACGTGTTCTAGA  4680
  F  F  G  D  A  A  E  Q  G  L  Y  K  D  I  W  A  R  V  L  D   448
TGTGGCTTATGAGGCAAAAGATGTCATAGATTCAATTATTGTTCGAGATAATGGTCTCTT  4740
  V  A  Y  E  A  K  D  V  I  D  S  I  I  V  R  D  N  G  L  L   468
ACATCTTATTTTCTCACTTCCCATTACCATAAAGAAGATCAAACTTATCAAAGAAGAGAT  4800
  H  L  I  F  S  L  P  I  T  I  K  K  I  K  L  I  K  E  E  I   488
```

FIGURE 5b

```
CTCTGCTTTAGATGAGAACATTCCCAAGGACAGAGGTCTAATCGTTGTGAACTCTCCCAA  4860
  S  A  L  D  E  N  I  P  K  D  R  G  L  I  V  V  N  S  P  K   508
GAAACCAGTTGAGAGAAAGTCATTGACAACTGATAAAATAATTGTAGGTTTTGAGGAGGA  4920
  K  P  V  E  R  K  S  L  T  T  D  K  I  I  V  G  F  E  E  E   528
GACAAACTTGATACTTAGAAAGCTCACCAGTGGACCCGCAGATTTAGATGTCATTTCGAT  4980
  T  N  L  I  L  R  K  L  T  S  G  P  A  D  L  D  V  I  S  I   548
CACCGGTATGCCGGGTTCAGGTAAAACTACTTTGGCATACAAAGTATACAATGATAAGTC  5040
  T  G  M  P  G  S  G  K  T  T  L  A  Y  K  V  Y  N  D  K  S   568
AGTTTCTAGACATTTTGACCTTCGTGCATGGTGCACGGTCGATCAAGGATATGACGACAA  5100
  V  S  R  H  F  D  L  R  A  W  C  T  V  D  Q  G  Y  D  D  K   588
GAAGTTGTTGGATACAATTTTCAGTCAAGTTAGTGGCTCAGATTCAAATTTGAGTGAGAA  5160
  K  L  L  D  T  I  F  S  Q  V  S  G  S  D  S  N  L  S  E  N   608
TATTGATGTTGCTGATAAATTGCGGAAACAACTGTTTGGAAAGAGGTATCTTATTGTCTT  5220
  I  D  V  A  D  K  L  R  K  Q  L  F  G  K  R  Y  L  I  V  L   628
AGATGATGTGTGGGATACTACTACATTGGATGAGTTGACAAGACCTTTTCCTGAAGCTAA  5280
  D  D  V  W  D  T  T  T  L  D  E  L  T  R  P  F  P  E  A  K   648
GAAAGGAAGTAGGATTATTTTGACAACTCGAGAAAAGGAAGTGGCTTTGCATGGAAAGCT  5340
  K  G  S  R  I  I  L  T  T  R  E  K  E  V  A  L  H  G  K  L   668
GAACACTGATCCTCTTGACCTTCGATTGCTAAGACCAGATGAAAGTTGGGAACTTTTAGA  5400
  N  T  D  P  L  D  L  R  L  L  R  P  D  E  S  W  E  L  L  E   688
GAAAAGGACATTTGGTAATGAGAGTTGCCCTGATGAACTATTAGATGTCGGTAAAGAAAT  5460
  K  R  T  F  G  N  E  S  C  P  D  E  L  L  D  V  G  K  E  I   708
AGCCGAAAATTGTAAAGGGCTTCCTTTGGTGGCTGATCTGATTGCTGGAGTCATTGCTGG  5520
  A  E  N  C  K  G  L  P  L  V  A  D  L  I  A  G  V  I  A  G   728
GAGGGAAAAGAAAAGGAGTGTGTGGCTTGAAGTTCAAAGTAGTTTGAGTTCTTTTATTTT  5580
  R  E  K  K  R  S  V  W  L  E  V  Q  S  S  L  S  S  F  I  L   748
GAACAGTGAAGTGGAAGTGATGAAAGTTATAGAATTAAGTTATGACCATTTACCACATCA  5640
  N  S  E  V  E  V  M  K  V  I  E  L  S  Y  D  H  L  P  H  H   768
CCTCAAGCCATGCTTGCTTCACTTTGCAAGTTGGCCGAAGGACACTCCTTTGACAATCTA  5700
  L  K  P  C  L  L  H  F  A  S  W  P  K  D  T  P  L  T  I  Y   788
TTTGTTGACTGTTTATTTGGGTGCTGAAGGATTTGTGGAAAAGACGGAGATGAAGGGTAT  5760
  L  L  T  V  Y  L  G  A  E  G  F  V  E  K  T  E  M  K  G  I   808
AGAAGAAGTGGTGAAGATTTATATGGATGATTTAATTTCCAGTAGCTTGGTAATTTGTTT  5820
  E  E  V  V  K  I  Y  M  D  D  L  I  S  S  S  L  V  I  C  F   828
CAATGAGATAGGTGATATACTGAATTTCCAAATTCATGATCTTGTGCATGACTTTTGTTT  5880
  N  E  I  G  D  I  L  N  F  Q  I  H  D  L  V  H  D  F  C  L   848
GATAAAAGCAAGAAAGGAAAATTTGTTTGATCGGATAAGATCAAGTGCTCCATCAGATTT  5940
  I  K  A  R  K  E  N  L  F  D  R  I  R  S  S  A  P  S  D  L   868
GTTGCCTCGTCAAATTACCATTGATTATGATGAGGAGGAGGAGCACTTTGGGCTTAATTT  6000
  L  P  R  Q  I  T  I  D  Y  D  E  E  E  E  H  F  G  L  N  F   888
TGTCATGTTCGATTCAAATAAGAAAAGGCATTCTGGTAAACACCTCTATTCTTTGAGGAT_ 6060
  V  M  F  D  S  N  K  K  R  H  S  G  K  H  L  Y  S  L  R  I   908
AAATGGAGACCAGCTGGATGACAGTGTTTCTGATGCATTTCACCTAAGACACTTGAGGCT  6120
  N  G  D  Q  L  D  D  S  V  S  D  A  F  H  L  R  H  L  R  L   928
TATTAGAGTGTTGGACCTGGAACCCTCTTTAATCATGGTGAATGATTCTTTGCTGAATGA  6180
  I  R  V  L  D  L  E  P  S  L  I  M  V  N  D  S  L  L  N  E   948
AATATGCATGTTGAATCATTTGAGGTACTTAAGAATTCGGACACAAGTTAAATATCTGCC  6240
  I  C  M  L  N  H  L  R  Y  L  R  I  R  T  Q  V  K  Y  L  P   968
TTTCTCTTTCTCAAACCTCTGGAATCTAGAAAGTCTGTTTGTGTCTAACAAAGGATCAAT  6300
  F  S  F  S  N  L  W  N  L  E  S  L  F  V  S  N  K  G  S  I   988
CTTGGTACTATTACCGAGAATTTTGGATCTTGTAAAGTTGCGAGTGCTGTCCGTGGGTGC  6360
  L  V  L  L  P  R  I  L  D  L  V  K  L  R  V  L  S  V  G  A  1008
```

FIGURE 5c

```
TTGTTCTTTCTTTGATATGGATGCAGATGAATCAATATTGATAGCAAAGGACACAAAGTT  6420
  C  S  F  F  D  M  D  A  D  E  S  I  L  I  A  K  D  T  K  L  1028
AGAGAACTTGAGAATATTAGGGGAACTGTTGATTTCCTATTCGAAAGATACAATGAATAT  6480
  E  N  L  R  I  L  G  E  L  L  I  S  Y  S  K  D  T  M  N  I  1048
TTTCAAAAGGTTTCCCAATCTTCAGGTGCTTCAGTTTGAACTCAAGGAGTCATGGGATTA  6540
  F  K  R  F  P  N  L  Q  V  L  Q  F  E  L  K  E  S  W  D  Y  1068
TTCAACAGAGCAACATTGGTTCCCGAAATTGGATTGCCTAACTGAACTAGAAACACTCTG  6600
  S  T  E  Q  H  W  F  P  K  L  D  C  L  T  E  L  E  T  L  C  1088
TGTAGGTTTTAAAAGTTCAAACACAAACCACTGTGGGTCCTCTGTTGCGACAAATCGGCC  6660
  V  G  F  K  S  S  N  T  N  H  C  G  S  S  V  A  T  N  R  P  1108
GTGGGATTTTCACTTCCCTTCAAATTTGAAAGAACTGTTGTTGTATGACTTTCCTCTGAC  6720
  W  D  F  H  F  P  S  N  L  K  E  L  L  L  Y  D  F  P  L  T  1128
ATCCGATTCACTATCAACAATAGCGAGACTGCCCAACCTTGAAAATTTGTCCCTTTATGA  6780
  S  D  S  L  S  T  I  A  R  L  P  N  L  E  N  L  S  L  Y  D  1148
TACAATCATCCAGGGAGAAGAATGGAACATGGGGGAGGAAGACACTTTTGAGAATCTCAA  6840
  T  I  I  Q  G  E  E  W  N  M  G  E  E  D  T  F  E  N  L  K  1168
ATTTTTGAACTTGCGTCTACTGACTCTTTCCAAGTGGGAGGTTGGAGAGGAATCCTTCCC  6900
  F  L  N  L  R  L  L  T  L  S  K  W  E  V  G  E  E  S  F  P  1188
CAATCTTGAGAAATTAAAACTGCAGGAATGTGGTAAGCTTGAGGAGATTCCACCTAGTTT  6960
  N  L  E  K  L  K  L  Q  E  C  G  K  L  E  E  I  P  P  S  F  1208
TGGAGATATTTATTCATTGAAATTTATCAAAATTGTAAAGAGTCCTCAACTTGAAGATTC  7020
  G  D  I  Y  S  L  K  F  I  K  I  V  K  S  P  Q  L  E  D  S  1228
TGCTCTCAAGATTAAGAAATACGCTGAAGATATGAGAGGAGGGAACGAGCTTCAGATCCT  7080
  A  L  K  I  K  K  Y  A  E  D  M  R  G  G  N  E  L  Q  I  L  1248
TGGCCAGAAGAATATCCCCTTATTTAAGTAGCATTTTGGTTGAACTTTGCTTGGTGATAT  7140
  G  Q  K  N  I  P  L  F  K  ---                               1257
TGTATATGATTAAAATATCCTGTGATGAGATTCCTCTTAGTTTCTTTTAACAAAAAATAT  7200
AATTTTTATAAGTACACATATCGTTTGTTAATTTGTCCATTTGTGATTGCAAGTCACACA  7260
TGAGGTATGTTCGTATTATGGGTTTCAACTTGATCAGACGTAATTTTAAGATAAGTGCTT  7320
ATATGATGTTGCATGCCAGATGGAAGTGACTATGTGAAGTTTATATTTTAAACATTAATC  7380
TTGTATACCAAACTACTATTCCTATGCTATGTTGTTTGCCATTGTCGTTCTCTCTTTATT  7440
TTTTTTCTTTCCATTCACACACACATTAATTTTCTAGTAGACCGCATATTACTACATCTG  7500
TATTGTCCGTATACAAGACGAATCCAGGATTTGATGTTTACAAGTATTTGTGAAGAATCC  7560
AGGATTTGATGTTTACAAGACAATTAGATTCATATATGTATAGGATTTTGACAGAAACTG  7620
AGGGATTCACATGACAATTACTCTGTGGATTTGCCTTTGGCTGTCCAAACCTCCTTTGTG  7680
TCTAACTTCGTCTGAAGTCCCATTTATATGCTCAAAGCTCAGTCAAGGTACTGATTCAAA  7740
AGCTAGGCTGTGAAGTAAACTTTAAAATGATATTGCTGCAAAGTCGCTCAACAAAGGGTC  7800
ATAACCATCACTACAACTACACAAGCTCAAGCAAGTAAACGCGGGTGAAAGATTAACATA  7860
GATCGCTATCCCCTGCAAAAGCTAAGGAAAGCATCTCTAACTTCTTAGCATGTACTCAAA  7920
CACACGATCTGTAAGGATGCCAGAAAGAGAAAGTTACGTTGCCGCAATTCCTTACAGTGT  7980
TGCACAATGTCCCCAAAACCAACATCACACTACAAAAAAAGGCTCAAATTCTGGGGGTTA  8040
TAATTAGACGGTCAATAACCCCTGCAATTTAGTGTTGTGGAGGTTGAATAAACTCCTCCA  8100
ATTAGGAGTGTCACAATTAAGTCGCGTGGGATTCTTGGCACATCCCGGTAAGGTTAACTA  8160
GCGGGGGTTTTGAACCCCAACCGCATTTCAAACTAGGAGTCGAAACCCCAACGATTTGTG  8220
AACTCGGGGGAGTCAAAAACCCCCGCAATAAATGATTTTTACATTAAAATTAATAGGAGC  8280
TTGGACCCCTGTGATTTATGAAATATAACTTTTTGTAGCATTTGCCAGAAATATTCAATT  8340
TTAGATACTAATAATAAATTAATTAACTAACATGTGCATCATTATTCAAAGGACATATTA  8400
GTATTAAGAAATAATACAATATTCAACACAAAAGTACCCAAACTCAAGATAGGATCAGTT  8460
TATGGAACTTCAACTAGTTTCACTATAATTATTGTCACTAACATCAGCTGGCTGCAAAGG  8520
AGAATACATAATAAGTGACTTTATCCAAACTCAAAATCATGGCTGAATGTAGTAAAACAC  8580
CAAAGATTATAATAATTTCCATTAATTATCATATACTACACAACAACAAACTTAAAACAA  8640
TATAGAAAAGGATTAAACCATTTACACAAGCAATGATTCTATACCATTTCAAAACGACAA  8700
CATACTGTACTACTAAACAAGACACCATCAAACTGATTTGGACAAATATTAACAATAGTT  8760
```

FIGURE 5d

```
AAAACATGAACAAAGAATCTCAGGTTTCTTGTCAGTAGAAAAGAGACAGACTAGGAACTG   8820
GAGTGCTATTTTTCTTATAAGAGACAATTAATGTTTACTTCTTTATATTTTGACTATAAG   8880
TTGATTGGTTATAATGTTTACGAGGTTGTATATAATCCGATGTTCAATGATATGACTTTC   8940
CTATTGACTGAAATGCTTGAACGCAAACAGTATATCTAGATTAAGAATGAGGACGAATTA   9000
CCTCTAGAGGCATGGGTAATGGAAGCATAACTCCTTGATAATGGTTGTTAGCCCACTGCA   9060
AGTCACAAAACAAAACATCCGTAATATTAACATACTAAGGTTGTAAGCACTAAACGACAA   9120
CAACTATGCCTCAATCCCAACTAAGTTGGAATCGACTATATGAATACTCACAATTTCGAT   9180
TTATAGACAAAGATACTAGTAGAAATGACGTCTTTCCTTTCTATGTTAACACTTGGACAG   9240
AGAATGTTAAAGACTTACAACAACAGAAAAGAGTTAAAATCATTTAATTGAGCAAGGATT   9300
TCAAAACGACAACACAATATACTCAATTTTTCGACGGAAACAACTGGTTGGACAACAGTG   9360
CTATTTGTAACTCCAATGAACAACACTGCAACGTACATGTATCTCATTGCACTAAATAAA   9420
TCCCGTTGAGAGTAACATATCAATAGTTACGAACAATATGATCACGACAAAGGATTGTAA   9480
GTACCACAGGACAAGTCATGCTTGCATGAAAAACGGATATGTAAAGAACCAAAATCCTGC   9540
TGCTGAAATAAGCAGTTATGATTATCCAAAAATCATGAATACACATGCACTTGAGTTTGT   9600
TCCAAGAAAAACACAACCAACTACTGTCGCAAGTGAAGATTCAAAAGTGACTATTGATGT   9660
TAATTCTTCCACAAGGTTGAATAATTTTGTCACTATAGGATTTAAGACGAAGAAGAAACA   9720
GGCGACAATTTTGTAAGCATAGACCTTCTTATGCAACTATGAGCTGGTATGCTATTCATT   9780
TTCTTTACTCGTAAAAATCGTTGATACTAAAGAATGCCAATCCAGTCCTGCTGAATAGGC   9840
GCCAGGTGACTGGTTGCTGTTAATAATTTT-3'                              9870
```

FIGURE 5e

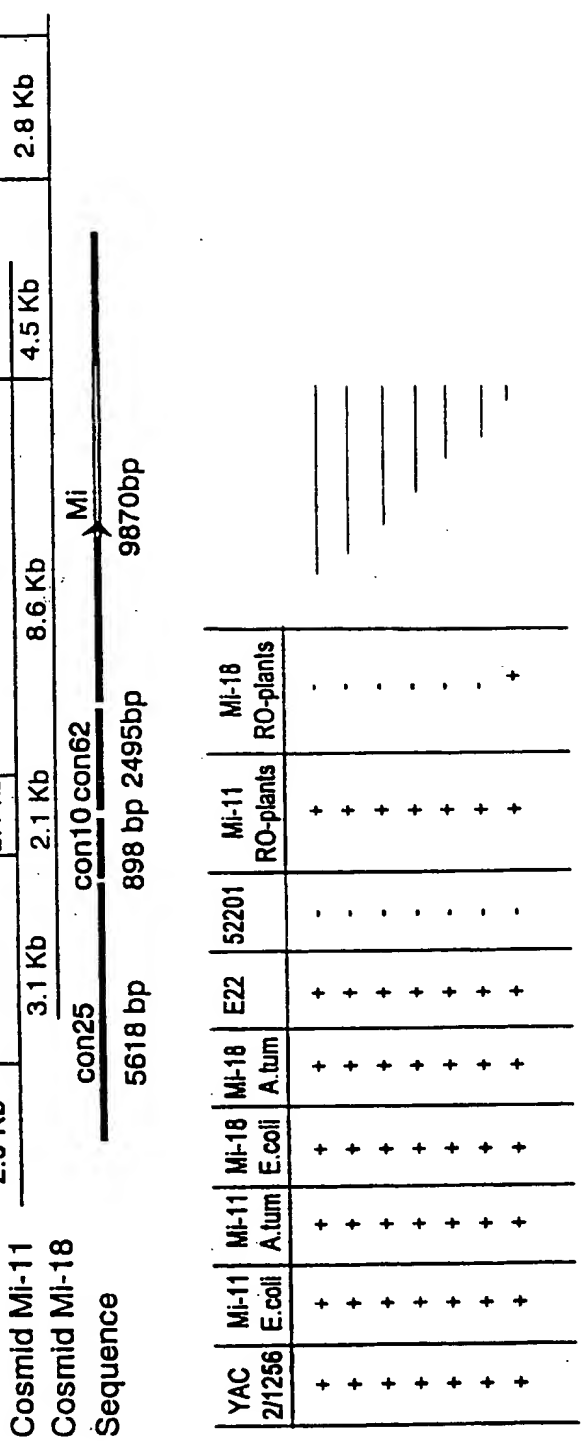
PM25
PM14
P
P
P
P
2.8 Kb
2.5 Kb
4.5 Kb
8.6 Kb
8.6 Kb
2.1 Kb
2.1 Kb
4.7 Kb
2.9 Kb
Cosmid Mi-11
Cosmid Mi-18
Sequence
3.1 Kb
con25
con10 con62
Mi
5618 bp
898 bp 2495bp
9870bp
YAC 2/1256
Mi-11 E.coli
Mi-11 A.tum
Mi-18 E.coli
Mi-18 A.tum
E22
52201
Mi-11 RO-plants
Mi-18 RO-plants

FIGURE 6

```
MEKRKDIEEA NNSLVLFSAL SKDIANVLIF LENEENQKAL DKDQVEKLKL KMAFICTYVQ
LSYSDFEQFE DIMTRNRQEV ENLLQSLLDD DVLTSLTSNM DDCISLYHRS YKSDAIMMDE
QLDFLLLNLY HLSKHHAEKI FPGVTQYEVL QNVCGNIRDF HGLILNGCIK HEMVENVLPL
FQLMAERVGH FLWEDQTDED SRLSELDEDE HNDRDSRLFQ LTHLLLKIVP TELEVMHICY
TNLKASTSAE VGRFIKKLLE TSPDILREYI IQLQEHMLTV IPPSTLGARN IHVMMEFLLL
ILSDMPKDFI HHDKLFDLLA HVGTLTREVS TLVRDLEEKL RNKEGNNQTN CATLDLLENI
ELLKKDLKHV YLKAPNSSQC CFPMSDGPLF MHLLHMHLND LLDSNAYSIS LIKEEIELVS
QELEFIRSFF GDAAEQGLYK DIWARVLDVA YEAKDVIDSI IVRDNGLLHL IFSLPITIKK
IKLIKEEISA LDENIPKDRG LIVVNSPKKP VERKSLTTDK IIVGFEEETN LILRKLTSGP
ADLDVISITG MPGSGKTTLA YKVYNDKSVS RHFDLRAWCT VDQGYDDKKL LDTIFSQVSG
SDSNLSENID VADKLRKQLF GKRYLIVLDD VWDTTTLDEL TRPFPEAKKG SRIILTTREK
EVALHGKLNT DPLDLRLLRP DESWELLEKR TFGNESCPDE LLDVGKEIAE NCKGLPLVAD
LIAGVIAGRE KKRSVWLEVQ SSLSSFILNS EVEVMKVIEL SYDHLPHHLK PCLLHFASWP
KDTPLTIYLL TVYLGAEGFV EKTEMKGIEE VVKIYMDDLI SSSLVICFNE IGDILNFQIH
DLVHDFCLIK ARKENLFDRI RSSAPSDLLP RQITIDYDEE EEHFGLNFVM FDSNKKRHSG
KHLYSLRING DQLDDSVSDA FHLRHLRLIR VLDLEPSLIM VNDSLLNEIC MLNHLRYLRI
RTQVKYLPFS FSNLWNLESL FVSNKGSILV LLPRILDLVK LRVLSVGACS FFDMDADESI
LIAKDTKLEN LRILGELLIS YSKDTMNIFK RFPNLQVLQF ELKESWDYST EQHWFPKLDC
LTELETLCVG FKSSNTNHCG SSVATNRPWD FHFPSNLKEL LLYDFPLTSD SLSTIARLPN
LENLSLYDTI IQGEEWNMGE EDTFENLKFL NLRLLTLSKW EVGEESFPNL EKLKLQECGK
LEEIPPSFGD IYSLKFIKIV KSPQLEDSAL KIKKYAEDMR GGNELQILGQ KNIPLFK
//
```

FIGURE 7A

MAFICTYVQL SYSDFEQFED IMTRNRQEVE NLLQSLLDDD VLTSLTSNMD DCISLYHRSY
KSDAIMMDEQ LDFLLLNLYH LSKHHAEKIF PGVTQYEVLQ NVCGNIRDFH GLILNGCIKH
EMVENVLPLF QLMAERVGHF LWEDQTDEDS RLSELDEDEH NDRDSRLFQL THLLLKIVPT
ELEVMHICYT NLKASTSAEV GRFIKKLLET SPDILREYII QLQEHMLTVI PPSTLGARNI
HVMMEFLLLI LSDMPKDFIH HDKLFDLLAH VGTLTREVST LVRDLEEKLR NKEGNNQTNC
ATLDLLENIE LLKKDLKHVY LKAPNSSQCC FPMSDGPLFM HLLHMHLNDL LDSNAYSISL
IKEEIELVSQ ELEFIRSFFG DAAEQGLYKD IWARVLDVAY EAKDVIDSII VRDNGLLHLI
FSLPITIKKI KLIKEEISAL DENIPKDRGL IVVNSPKKPV ERKSLTTDKI IVGFEEETNL
ILRKLTSGPA DLDVISITGM PGSGKTTLAY KVYNDKSVSR HFDLRAWCTV DQGYDDKKLL
DTIFSQVSGS DSNLSENIDV ADKLRKQLFG KRYLIVLDDV WDTTTLDELT RPFPEAKKGS
RIILTTREKE VALHGKLNTD PLDLRLLRPD ESWELLEKRT FGNESCPDEL LDVGKEIAEN
CKGLPLVADL IAGVIAGREK KRSVWLEVQS SLSSFILNSE VEVMKVIELS YDHLPHHLKP
CLLHFASWPK DTPLTIYLLT VYLGAEGFVE KTEMKGIEEV VKIYMDDLIS SSLVICFNEI
GDILNFQIHD LVHDFCLIKA RKENLFDRIR SSAPSDLLPR QITIDYDEEE EHFGLNFVMF
DSNKKRHSGK HLYSLRINGD QLDDSVSDAF HLRHLRLIRV LDLEPSLIMV NDSLLNEICM
LNHLRYLRIR TQVKYLPFSF SNLWNLESLF VSNKGSILVL LPRILDLVKL RVLSVGACSF
FDMDADESIL IAKDTKLENL RILGELLISY SKDTMNIFKR FPNLQVLQFE LKESWDYSTE
QHWFPKLDCL TELETLCVGF KSSNTNHCGS SVATNRPWDF HFPSNLKELL LYDFPLTSDS
LSTIARLPNL ENLSLYDTII QGEEWNMGEE DTFENLKFLN LRLLTLSKWE VGEESFPNLE
KLKLQECGKL EEIPPSFGDI YSLKFIKIVK SPQLEDSALK IKKYAEDMRG GNELQILGQK
NIPLFK
//

FIGURE 7B

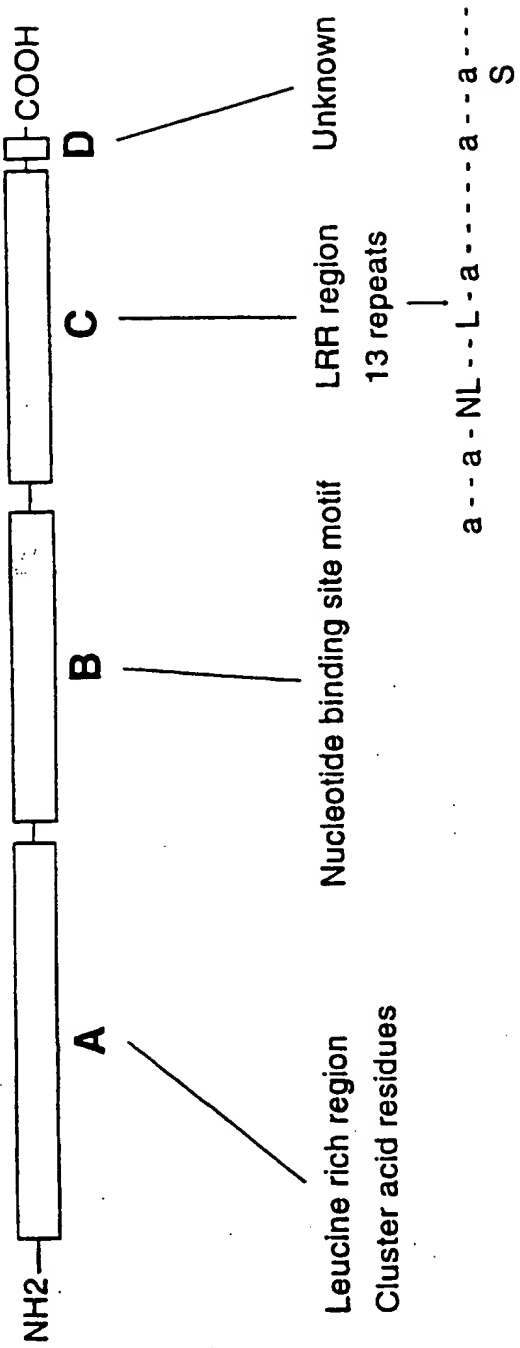
COOH
D
C
B
A
NH2
Unknown
LRR region
13 repeats
a - - a - NL - - L - a - - - - - a - - a - - -
S
Nucleotide binding site motif
Leucine rich region
Cluster acid residues

FIGURE 8

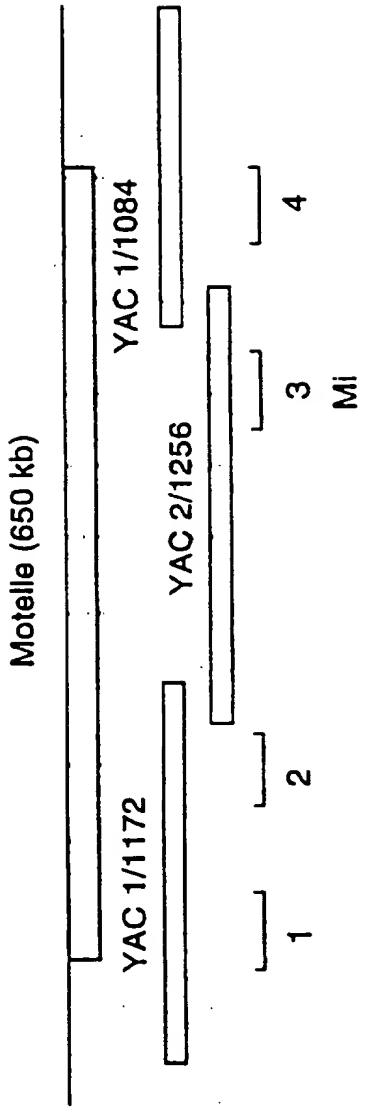

FIGURE 9

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(57) Abstract

The invention relates to genes capable of conferring resistance against nematodes and/or aphids. Preferred nucleic acids of the invention are DNA sequences which are at least part of the DNA sequence provided on figure 5 or homologous thereto. The invention further relates to vectors, cells and seeds comprising said nucleic acids, as well as genetically transformed plants which are resistant to nematodes and/or aphids. The invention also relates to oligonucleotides, primers, diagnostic kit and polypeptides.